

FORM PTO-590 (Rev. 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

## TRANSMITTAL LETTER TO THE UNITED STATES

1091-2 PCT/US

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/555780

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/FR98/02605

02 December 1998

03 December 1997

## TITLE OF INVENTION

Mixed Lipopeptide Micelles for Inducing an Immune Response and Their Therapeutic Uses

## APPLICANT(S) FOR DO/EO/US

Helene Gras-Masse, Marc Bossus, Guy Lippens, Jean-Michel Wieruszkeski, Andre Tartar, Jean-Gerard Guillet and Isabelle Bourgault-Villada

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

## Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Sequence Listing in Printed Form

Sequence Listing on Computer Diskette

U.S. APPLICATION NO. (IF KNOWN) <b>09/355780</b>	INTERNATIONAL APPLICATION NO. <b>PCT/FR98/02605</b>	ATTORNEY'S DOCKET NUMBER <b>1091-2 PCT/US</b>
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21. The following fees are submitted:				CALCULATIONS PTO USE ONLY	
<b>BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :</b>					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$970.00					
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$840.00					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$690.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$670.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$96.00					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>\$840.00</b>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				<b>\$0.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	x \$18.00	<b>\$72.00</b>	
Independent claims	1 - 3 =	0	x \$78.00	<b>\$0.00</b>	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$912.00</b>	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>SUBTOTAL =</b>				<b>\$912.00</b>	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				<b>\$0.00</b>	
<b>TOTAL NATIONAL FEE =</b>				<b>\$912.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				<b>\$0.00</b>	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$912.00</b>	
				Amount to be: refunded	\$
				charged	\$

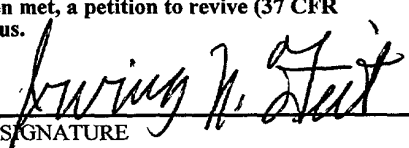
☒ A check in the amount of **\$912.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **08-2461** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

<p>Irving N. Feit, Esq. Hoffmann &amp; Baron, LLP 6900 Jericho Turnpike Syosset, New York 11791 United States of America</p> <p>Telephone: 516-822-3550 Facsimile: 516-822-3582</p>	<p> SIGNATURE</p> <p>Irving N. Feit NAME</p> <p>28,601 REGISTRATION NUMBER</p> <p>02 June 2000 DATE</p>
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Gras-Masse et al.

Examiner: Unassigned

Serial No.: Unassigned

Group Art Unit: Unassigned

Filed: Herewith

Docket: 1091-2 PCT/US

Int'l. Filing Date: December 2, 1998

Int'l. Appl. No.: PCT/FR98/02605

For: MIXED LIPOPEPTIDE  
MICELLES FOR INDUCING AN  
IMMUNE RESPONSE AND  
THEIR THERAPEUTIC USES

Dated: June 1, 2000

BOX PCT  
Assistant Commissioner for Patents  
Washington, DC 20231  
Att: EO/US

PRELIMINARY AMENDMENT

Sir:

Prior to examining the above-identified National Phase PCT application, please amend the application as follows:

IN THE CLAIMS:

Please amend Claims 1-21 as follows:

1. (Amended) A composition of mixed [Mixed] micelles or micro-aggregates for inducing an immune response [containing at least] comprising:
  - a first lipopeptide comprising at least one CTL antigenic determinant and at least one lipid unit, and
  - a second lipopeptide comprising at least one helper T antigenic determinant and at least one lipid unit[, which may be of a different type from the first lipopeptide unit].

1 (Amended) A composition [Micelles or micro-aggregates] according to claim 1 [characterized in that] wherein the lipopeptides independently comprise one or more C<sub>4</sub>-C<sub>18</sub> lipid units.

3. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 and 2] Claim 1 [characterized in that] wherein the lipopeptides independently comprise one or two C<sub>4</sub>-C<sub>18</sub> lipid chains linked by a covalent bond to one or two amino acids of the peptide part.

4. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 3] Claim 1 [characterized in that] wherein the lipid units of the lipopeptides are composed of two palmitic acid chains linked to the NH<sub>2</sub> groups of a lysine.

5. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 4] Claim 1 [characterized in that] wherein the lipid units of the lipopeptides independently comprise a residue of palmitic acid, 2-aminohexadecanoic acid, oleic acid, linoleic acid, linolenic acid, pimelautide, trimexautide, or a derivative of cholesterol.

6. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 5] Claim 1 [characterized in that] wherein the non-lipid part of the lipopeptides, comprising the antigenic determinant, comprises between 10 and 100[, and preferably between 10 and 50] amino acids.

7. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 6] Claim 1 [characterized in that] wherein the helper T antigenic determinant is a multivalent antigenic determinant.

8. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 7] Claim 1 [characterized in that] wherein the helper T antigenic determinant is the peptide 830-843 of the tetanus toxin with the following sequence:

QYIKANSKFIGITE

9. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 7] Claim 1 [characterized in that] wherein the helper T antigenic determinant is the antigenic determinant of hemagglutinin or the PADRE antigenic determinant.

10. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 9] Claim 1 [characterized in that] wherein the lipopeptides comprise at least one CTL antigenic determinant selected from the group consisting of a specific protein of melanoma, of a protein from HIV, from HBV, from papillomavirus, or protein p53, or a specific protein of *Plasmodium falciparum*.

11. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 10] Claim 1 [characterized in that they] wherein said micelles or micro-aggregates comprise the following lipopeptides:

GAG 17	EKIRLRPGGKKKYKLKHIVK(Pam)-NH <sub>2</sub>
GAG 253	NPPIPVGEIYKRWILGLNKIVRMYSPTSILDK(Pam)-NH <sub>2</sub>
POL 325	AIFQSSMTKILEPFRKQNPDIVIYQYMDDLKY(Pam)-NH <sub>2</sub>
NEF 66	VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLK(Pam)-NH <sub>2</sub>
NEF 116	HTQGYFPDWQNYTPGPGVRYPLTFGWLYKLLK(Pam)-NH <sub>2</sub>
TT	Ac-QYIKANSKFIGITELKKK(Pam)-NH <sub>2</sub>

12. (Amended) A composition [Micelles or micro-aggregates] according to [one of claims 1 to 10] Claim 1 [characterized in that they] wherein said micelles or micro-aggregates comprise the following lipopeptides:

LSA3 CT1	LLSNIEEPKENIIDNLLNNIK(Pam)-NH <sub>2</sub>
LSA3 NR1	Ac-DELFNELLNSVDVNGEVKENILEESQK(Pam)-NH <sub>2</sub>
LSA3 NRII	Ac-LEESQVNDIDFNSLVKSVQQEQQHNVK(Pam)-NH <sub>2</sub>
LSA3 RE	K(Pam)VESVAPSVEESVAPSVEESVAENVEESVAENV-NH <sub>2</sub>

13. (Amended) A method [Use of micelles or micro-aggregates according to one of claims 1 to 12] for the production of a drug or a vaccine for inducing a specific immune response comprising micelles or micro-aggregates according to Claim 1.

14. (Amended) A method according to Claim 13 wherein said [Use of micelles or micro-aggregates according to one of claims 1 to 12 for the production of a drug or a vaccine for inducing a] specific immune response is against HIV, HBV, papillomavirus, p53, melanoma or malaria induced by *Plasmodium falciparum*.

15. (Amended) A pharmaceutical [Pharmaceutical] composition [characterized in that it comprises] comprising a pharmacologically effective dose of micelles or micro-aggregates according to [one of claims 1 to 12] Claim 1 and pharmaceutically compatible vehicles.

16. (Amended) A drug [Drug] or vaccine [characterized in that it comprises] comprising micelles or micro-aggregates according to [one of claims 1 to 12] Claim 1.

17. (Amended) A method [Method] for producing micelles or micro-aggregates according to [one of claims 1 to 12] Claim 1, comprising the following steps:

- [dispersion of] dispersing each of the constituent lipopeptides in a solution of concentrated acetic acid of about 80% concentration then
- mixing the solutions thus obtained.

18. (Amended) A method [Method] according to Claim 17 [characterized in that] wherein the [production of a dispersion] dispersing of the lipopeptides dissolved in acetic acid is controlled by [the] a two-dimensional nuclear magnetic resonance method.

19. (Amended) A method [Method] for inducing an immune response against a particular antigen comprising at least the administration of micelles or micro-aggregates according to [one of claims 1 to 12] Claim 1 to an individual for whom such a response is desired.

20. (Amended) A method [Method] of immunization against a pathogenic agent comprising the administration of micelles or micro-aggregates according to [one of claims 1 to 12] Claim 1 to an individual for whom such an immunization is sought.

21. (Amended) A method [Method] according to [one of claims 19 and 20] Claim 19, [characterized in that] wherein the pathogenic agent is HIV, HBV, papillomavirus, melanoma or *plasmodium falciparum*, and wherein the antigen is an antigen of one of [these] said pathogenic agents, or p53.

Please add the following new claims:

--22. A composition according to Claim 1, wherein the at least one lipid unit in the second lipopeptide is different from the at least one lipid unit in the first lipopeptide.

23. A composition according to Claim 6, wherein the non-lipid part of the lipopeptides, comprising the antigenic determinants, comprises between 10 and 50 amino acids.

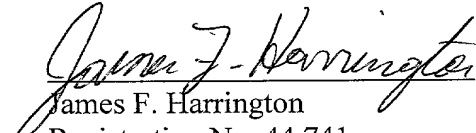
24. A method according to Claim 20, wherein the pathogenic agent is HIV, HBV, papillomavirus, melanoma, or *Plasmodium falciparum*, and wherein the antigen is an antigen of one of said pathogenic agents, or p53.--

#### REMARKS

Applicant respectfully requests entry of this Preliminary Amendment prior to examination on the merits of the National Stage PCT Application filed herewith. This Amendment rewrites the claims in a more traditional U.S. format and removes multiple dependencies. The new claims are dependent claims that contain matter included in the claims as originally filed. No new matter has been added.

Accordingly, this case is believed to be in all respects in condition for examination on the merits, and such examination and favorable consideration are respectfully and earnestly solicited.

Respectfully submitted,

  
James F. Harrington  
Registration No. 44,741  
Attorney for Applicants

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6900 Jericho Turnpike  
Syosset, New York 11791  
(516) 822-3550  
JFH:jp

113005\_1.DOC



## SEQUENCE LISTING

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 GRAS-MASSE, Hélène  
 BOSSUS, Marc  
 LIPPENS, Guy  
 WIERUSZESKI, Jean-Michel  
 TARTAR, André  
 GUILLET, Jean-Gérard  
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<222> (1)

<223> ACETYLTATION

<400> 36

Leu Glu Glu Ser Gln Val Asn Asp Asp Ile Phe Asn Ser Leu Val Lys  
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Ser Val Gln Gln Glu Gln Gln His Asn Val Lys  
20 25

<210> 37

<211> 33

<212> PRT

<213> Plasmodium falciparum

<220>

<221> LIPID

<222> (1)

<223> PALMITOYL DERIVATIVE

<400> 37

Lys Val Glu Ser Val Ala Pro Ser Val Glu Glu Ser Val Ala Pro Ser  
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Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn  
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Val

<210> 38

<211> 9

<212> PRT

<213> Homo sapiens

<400> 38  
 Glu Asp Ala Glu Leu Asn Pro Arg Phe  
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 Asp Glu Leu Glu Ala Val Pro Asn Ile  
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<400> 41  
 Lys Glu Asp Ala Leu Gln Arg Pro Val  
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<210> 42  
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<400> 42  
 Glu Asp Ala Leu Gln Arg Pro Val Ala  
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<210> 43  
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<400> 43  
 Gly Glu Lys Leu Arg Val Leu Gly Tyr  
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Glu Asp Thr Met Glu Val Glu Glu Phe
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<400> 45  
Met Glu Tyr Leu Glu Lys Lys Asn Phe  
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Asn Glu Glu Ala Ala Asp Glu Val Phe  
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Leu Phe Gln Lys Leu Ala Ser Gln Leu
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<212> PRT
<213> Homo sapiens
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Phe Leu Asn Val Ile Val His Ser Ala
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<210> 76

<211> 9

<212> PRT

<213> Homo sapiens

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Val Leu Leu Tyr Met Ala Thr Gln Ile

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<210> 77

<211> 9

&lt;212&gt; PRT

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Phe Ile Pro Leu Ile Ser Thr Arg Val

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<210> 78

<211> 9

&lt;212&gt; PRT

<213> Homo sapiens

<400> 78

Val Val Leu Asp Ser Thr Glu Ala Leu

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<210> 79

<211> 9

&lt;212&gt; PRT

<213> Homo sapiens

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Asn Gln Glu Arg Phe Arg Met Ile Tyr

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<210> 80

<211> 9

<212> PRT

<213> Homo sapiens

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Val Gly Asp Ala Ser Arg Pro Pro Tyr

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<210> 81

<211> 10

<212> PRT

<213> Homo sapiens

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<211> 9

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<213> Homo sapiens

<400> 82

Lys Leu Ala Ser Gln Leu Gly Val Tyr  
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<210> 83

<211> 10

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<213> Homo sapiens

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<211> 9

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Ile Val His Ser Ala Thr Gly Phe Lys  
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<210> 85

<211> 9

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<213> Homo sapiens

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Ala Thr Gly Phe Lys Gln Ser Ser Lys  
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<210> 86

<211> 9

<212> PRT

<213> Homo sapiens

<400> 86

Lys Gln Ser Ser Lys Ala Leu Gln Arg

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<400> 87  
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<210> 88  
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<400> 88  
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<210> 89  
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<400> 89  
 Asn Leu Phe Ser Ala Leu Ile Lys Lys  
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<210> 90  
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 Thr Val Ala Pro Ala Ser Gly Leu Pro His Lys  
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<210> 91  
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<210> 92  
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<400> 93  
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 Arg Val Glu Gly Asn Leu Ala Arg Val Glu Tyr  
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<210> 98  
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<400> 100  
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<210> 101  
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 <212> PRT  
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<400> 101  
 Gly Leu Ala Pro Pro Gln His Leu Ile Arg Val  
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<210> 102  
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 <212> PRT  
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<400> 102  
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004400 005500 006600 007700 008800 009900

<400> 103  
Pro Leu Asp Gly Glu Tyr Phe Thr Leu  
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Arg Val Arg Ala Met Ala Ile Tyr Lys
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Arg Arg Thr Glu Glu Glu Asn Leu Arg  
1 5

<400> 106  
Glu Leu Pro Pro Gly Ser Thr Lys Arg  
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<210> 108





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<210> 119  
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 <212> PRT  
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<400> 121  
 Thr Leu Gly Ile Val Cys Pro Ile  
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<210> 122  
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<400> 122  
 Thr Ile His Asp Ile Ile Leu Glu Cys Val  
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<400> 123  
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<210> 124  
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<212> PRT  
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<400> 124  
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<210> 125  
 <211> 23  
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<400> 125  
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Tyr Arg Asp Gly Asn Pro Tyr  
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<210> 127  
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Gly Arg Trp Thr  
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<210> 128  
 <211> 9  
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 Gln Ala Glu Pro Asp Arg Ala His Tyr  
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<400> 129  
Glu Pro Asp Arg Ala His Tyr Asn Ile Val  
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<400> 130  
Gly Leu Glu Gly Leu Ile His Ser Gln Arg Arg  
1 5 10

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Phe Pro Asp Trp Gln Asn Tyr Thr  
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Leu Thr Phe Gly Trp Cys Tyr Lys Leu  
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<210> 135  
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Ser Leu Tyr Asn Thr Val Ala Thr Leu  
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Ala Leu Val Glu Ile Cys Thr Glu Met  
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<210> 138  
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<400> 138  
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<210> 139  
<211> 9  
<212> PRT  
<213> Human immunodeficiency virus type 1

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Lys Ile Tyr Gln Tyr Met Asp Asp Leu  
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<400> 140  
Lys Ile Glu Glu Leu Arg Gln His Leu  
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Leu Leu Arg Trp Gly Leu Thr Thr Pro Asp Lys
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Ile Leu Lys Glu Pro Val His Gly Val  
1 5

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Pro Leu Val  Lys  Leu Trp Tyr  Gln Leu
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Glu Leu Val Asn Gln Asp Glu Gln Leu  
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<210> 145

<400> 145  
Pro Leu Thr Phe Gly Trp Cys Phe Lys Leu  
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Val Leu Gln Trp Arg Phe Asp Ser Arg Leu
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<400> 147  
Ala Leu His His Val Ala Arg Glu Leu  
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Ser Leu Leu Asn Ala Thr Val Asp Ile  
1 5

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Asp Leu Asn Thr Met Leu Asn Thr Val  
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<210> 150
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<210> 151  
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<211> 8  
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Thr Val Tyr Tyr Gly Val Pro Val  
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<400> 155  
Arg Ile Gln Arg Gly Pro Gly Arg Ala  
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<210> 156  
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 <212> PRT  
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<400> 156  
 Thr Leu Thr Ser Cys Asn Thr Ser Val  
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<210> 164  
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 <212> PRT  
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<210> 165  
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<210> 166  
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<400> 166

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Ala Ile Phe Gln Ser Ser Met Thr Lys  
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<400> 167  
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<210> 168  
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<210> 169  
<211> 10  
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<400> 169  
Thr Val Tyr Tyr Gly Val Pro Val Trp Lys  
1 5 10

<210> 170  
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<400> 170  
Arg Leu Arg Asp Leu Leu Leu Ile Val Thr Arg  
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<210> 171  
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<400> 171  
Lys Ile Arg Leu Arg Pro Gly Gly Lys  
1 5

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<400> 172  
Ala Ile Phe Gln Ser Ser Met Thr Lys  
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<400> 173  
Gln Ile Tyr Gln Glu Pro Phe Lys Asn Leu Lys  
1 5 10

<400> 174  
Gln Val Pro Leu Arg Pro Met Thr Tyr Lys  
1 5 10

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<400> 175
Ala Val Asp Leu Ser His Phe Leu Lys
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<400> 176  
Ala Cys Gln Val Gly Gly Pro Gly His Lys  
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Ala Thr Leu Tyr Cys Val His Gln Arg  
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<210> 178

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<213> Human immunodeficiency virus type 1

<400> 178

Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr  
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<210> 179

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Arg Tyr Leu Lys Asp Gln Gln Leu  
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<210> 180

<211> 8

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Tyr Leu Lys Asp Gln Gln Leu Leu  
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<210> 181

<211> 9

<212> PRT

<213> Human immunodeficiency virus type 1

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Val Tyr Tyr Asp Pro Ser Lys Asp Leu  
1 5

<210> 182

<211> 9

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<213> Human immunodeficiency virus type 1

<400> 182

Ile Tyr Gln Glu Pro Phe Lys Asn Leu

00277 002550

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<400> 183  
Glu Ser Glu Leu Val Asn Gln Ile Ile Glu Gly  
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Glu Thr Ile Asn Glu Glu Ala Ala Glu Trp  
1 5 10

<400> 185  
Glu Val Ile Pro Met Phe Ser Ala Leu  
1 5

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Arg Ala Ile Arg His Ile Pro Arg Arg  
1 5

<400> 187  
Arg Leu Arg Asp Leu Leu Leu Ile Val Thr Arg  
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<210> 188  
 <211> 9  
 <212> PRT  
 <213> Human immunodeficiency virus type 1

<400> 188  
 Arg Ile Lys Gln Ile Ile Asn Met Trp  
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<210> 189  
 <211> 9  
 <212> PRT  
 <213> Human immunodeficiency virus type 1

<400> 189  
 His Arg Leu Arg Asp Leu Leu Leu Ile  
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<210> 190  
 <211> 10  
 <212> PRT  
 <213> Human immunodeficiency virus type 1

<400> 190  
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<210> 191  
 <211> 10  
 <212> PRT  
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<400> 191  
 Ile Ile Leu Gly Leu Asn Lys Ile Val Arg  
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<210> 192  
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 <212> PRT  
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<400> 192  
 Tyr Leu Ala Trp Val Pro Ala His Lys  
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<210> 193  
 <211> 10  
 <212> PRT  
 <213> Human immunodeficiency virus type 1

0995760 11700



<400> 193

Thr Pro Gly Pro Gly Val Arg Tyr Pro Leu  
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<210> 194

<211> 9

<212> PRT

<213> Human immunodeficiency virus type 1

<400> 194

Phe Pro Val Thr Gln Val Pro Leu Arg  
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<210> 195

<211> 10

<212> PRT

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<400> 195

Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile  
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<210> 196

<211> 9

<212> PRT

<213> Human immunodeficiency virus type 1

<400> 196

Ile Pro Arg Arg Ile Arg Gln Gly Leu  
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<210> 197

<211> 9

<212> PRT

<213> Human immunodeficiency virus type 1

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 $\langle 210 \rangle$  211

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&lt;212&gt; PRT

<213> Human immunodeficiency virus type 1

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<211> 9

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<211> 9
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<210> 284
<211> 14
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<400> 284

11

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**Mixed lipopeptide micelles for inducing an immune response and their  
therapeutic uses**

The present invention relates to mixed lipopeptide micelles for inducing an immune response.

5 A further object is the use of these micelles for therapeutic purposes.

There are two types of effector immune responses: the humoral response due to antibodies, and the cytotoxic response due to CD8<sup>+</sup> T lymphocytes.

An effective cytotoxic response requires the presentation of the antigens  
10 to the cytotoxic CD8<sup>+</sup> T lymphocytes (CTL), in combination with class I molecules of the Major Histocompatibility Complex (MHC), but also to helper CD4<sup>+</sup> T lymphocytes (HTL) in combination with class II MHC molecules.

The use of lipopeptides for inducing a cytotoxic response, in other words the in vivo generation of cytotoxic T lymphocytes, has already been described.  
15 In particular, application FR-90 15 870 published under the n° 2 670 787 (Institut Pasteur de Lille, Institut Pasteur, INSERM) discloses lipopeptides composed of a peptide portion comprising 10 to 40 amino acids and a lipid portion which may be derived from fatty acids or steroid groups.

These lipopeptides show a good aptitude for inducing a cytotoxic  
20 response. However it was advisable to make them able to induce a better quality response by addition of a helper T response whose importance for effective induction and maintenance of the cytotoxic response is known. It was also advisable to make them able to induce a response in as many individuals as possible.

25 BOURGAULT et al. (1994, J. Immunol., 2530-2537) induced a CTL and HTL response from a mixture of lipopeptides, in the form of an emulsion with an oily adjuvant.

Nevertheless, it was necessary to add incomplete Freund's adjuvant (IFA). The immunogenicity of the vaccine preparation used necessarily  
30 involved the functional co-presentation of the HTL and CTL units located in one or more lipopeptides in the mixture. However, the effectiveness of the co-

presentation of the different units involved depended on the combination with the incomplete Freund's adjuvant within a very fine emulsion.

An article under the name of VITIELLO et al. (1995, J. Clin. Invest., 95, 341-349) raised the possibility of inducing a CTL response in a selected human population (HLA-A2) by using a lipopeptide containing a sequential combination of a CTL HLA-A2 antigenic determinant and a multivalent helper (HTL) antigenic determinant. It should be noted that this study was carried out on a genetically restricted population.

This article also reports an experiment during which two types of associations between the HTL antigenic determinant and the CTL antigenic determinant were compared: on the one hand, a covalent sequential combination within the same lipopeptide, and on the other an association by simple mixture of a lipopeptide containing the CTL unit with a peptide containing the HTL unit. The results of this study showed a very clear advantage of the covalent combination compared to the mixture, as performed by the authors, in other words by mixture of solutions containing DMSO and PBS buffer (the peptides or lipopeptides were kept in stock solutions at a concentration of 10 - 20 mg/ml and diluted with PBS just before use).

However, the combination within the same lipopeptide molecule of the cytotoxic and helper antigenic determinants, although able to induce an effective immune response, required the synthesis of long amino acid sequences presenting the multiple antigenic determinants able to combine with several HLA or superfamilies of class I and class II HLA. The covalent combination of all these units within a single molecule presented technical problems not easily overcome, both from the points of view of synthetic methods and analytical characterisation.

In any case, this article mentions the combination of a lipopeptide and a peptide, and not of two lipopeptides. For this reason no mixed micelle formation could take place.

Another article, published by DON DIAMOND et al. (1997, Blood, 90, n° 5), mentions the immunogenicity of a mixture between a peptide carrying a minimal CTL antigenic determinant (pp 65, sequence 495-503 of the

cytomegalovirus matrix protein) and a dipalmitoyl peptide containing an HTL antigenic determinant. The mixture was achieved by mixing solutions in dilute acetic acid or in DMSO, using an ultrasonic treatment for 15 to 30 seconds.

This article thus does not describe a mixture of lipopeptides independently containing a CTL antigenic determinant and an HTL antigenic determinant, but the mixture of a lipopeptide containing an HTL antigenic determinant and a nonapeptide corresponding to a minimal CTL antigenic determinant. In addition, there is no mention of the formation of mixed micelles or of micro-aggregates. In this particular case, however, the possibility of direct combination between the nonapeptide and the class I MHC expressed at the surface of the cells could explain the success of the approach followed. The immunogenicity of the preparation indicates that there was effectively co-presentation of the HTL and CTL antigenic determinants by the same antigen-presenting cell; however, the minimal nonapeptide used has the capacity to link directly with the class I MHC at the surface of the antigen-presenting cell, without its presentation by the cell being necessary.

The authors conclude by recognising that there are still several obstacles to long-term immunity, which confirms the experimental character of this study.

The difficulty of obtaining an immune response depending on a double presentation of the peptides separately presenting the HTL and CTL antigenic determinants is now explained by a publication by STUHLER (1997, Proc. Natl. Acad. Sci. USA, 94, 622-627). To be able to observe the induction of a CTL response, it is absolutely necessary that the HTL and CTL antigenic determinants are present on the surface of the same antigen-presenting cell (APC) to be able to be recognised at the same time by the helper T cells recognising the HTL antigenic determinant and the cytotoxic T cells recognising the CTL antigenic determinant.

It follows from the above that compositions containing within the same micelles, or the same micro-aggregates, on the one hand lipopeptides presenting a CTL antigenic determinant and on the other lipopeptides

containing an auxiliary T antigenic determinant, i.e. mixed micelles or micro-aggregates, have never, to the knowledge of the applicant, been described.

However, as described above, it is absolutely necessary that the two antigenic determinants, cytotoxic and helper T, are present on the surface of  
5 the same antigen-presenting cell.

In addition to the necessity of a co-presentation of the two antigenic determinants on the surface of the same cell, it is also essential to solubilize the lipopeptides, so as to allow their administration to patients, and their sterilisation by filtration.

10 The applicant has thus endeavoured to find a solution to these different problems.

He has shown that, in order to obtain micelles individually formed from all the peptides present in the mixture, whether containing HTL or CTL antigenic determinants, it was necessary to combine the different lipopeptides  
15 after having previously dispersed them at the molecular level in a suitable solvent.

The object of the present invention is thus micelles or micro-aggregates for inducing an immune response containing at least :

- a first lipopeptide comprising at least one CTL antigenic determinant,  
20 or cytotoxic antigenic determinant, and at least one lipid unit, and
- a second lipopeptide comprising at least one helper antigenic determinant and at least one lipid unit, which may be of a different type from the first lipopeptide unit.

In the scope of the present invention, the expression "immune response"  
25 means the whole of the induced immune response, which includes the cytotoxic response and the humoral response.

The micelles according to the present invention are not limited to two lipopeptides, but may contain other lipopeptides independently presenting HTL or CTL antigenic determinants.

30 To understand the present invention, helper T antigenic determinant should be understood as meaning an amino acid sequence able to bind with at

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CTL antigenic determinant should be understood as meaning an amino acid sequence able to bind with at least one class I HLA receptor and able to be recognised by cytotoxic T lymphocytes.

In addition, by micelles or micro-aggregates should be understood aggregates of lipopeptides with a size making them able to be assimilated simultaneously by any antigen-presenting cell (APC) and preferably with a size less than about 1  $\mu\text{m}$ .

The mixed micelles according to the invention, in other words comprising lipopeptides containing cytotoxic antigenic determinants and lipopeptides containing helper T antigenic determinants, have the advantage of combining, within the same microvolume which can be assimilated by a single APC, a wide variety of CTL and HTL antigenic determinants, without their covalent combination being necessary, while respecting the required criterion of chemical definition. Micelles which each contain a single type of lipopeptide, containing a CTL antigenic determinant or an HTL antigenic determinant, do not result in an effective co-presentation corresponding to the induction of a strong effector response.

In addition, obtaining a CTL response by the use of mixed micro-aggregates or micelles avoids the use of emulsions with oily adjuvants, such as incomplete Freund's adjuvant, whose use is not approved in human therapeutics. The micelles and micro-aggregates according to the present invention are however compatible with the use of emulsions with clinically acceptable oily vehicles.

A further advantage of the mixed micro-aggregates or micelles according to the present invention, each containing at least two types of lipopeptides, is that the solubilization of lipopeptides with a low solubility in water or in clinically

acceptable solvents, or insoluble lipopeptides, may be improved by their combination with other lipopeptide(s) with better solubility.

The micelles according to the present invention also show the advantage, compared to lipopeptides in which the HTL and CTL units are combined covalently and whose size is limited, such as those described by VITIELLO et al. (1995, cited above), of allowing the combination of a wide variety of units, and thus can be used for the vaccination of human or animal populations not selected on the basis of genetic restriction.

The micelles according to the present invention may contain a lipopeptide with at least a CTL antigenic determinant and another lipopeptide containing at least a helper antigenic determinant. However, such micelles may also contain several different lipopeptides containing different cytotoxic antigenic determinants and different lipopeptides with different helper antigenic determinants.

The lipid units of the lipopeptides may independently be one or more C<sub>4</sub>-C<sub>18</sub> units, and in particular one or more C<sub>4</sub>-C<sub>18</sub> chains derived from fatty acids, or fatty alcohols, optionally branched and unsaturated or derived from a steroid.

They may contain one or two C<sub>4</sub> to C<sub>18</sub> lipid chains linked by a covalent bond to one or two amino acids of the peptide part. They may also be composed of two palmitic acid chains linked to the alpha and epsilon NH<sub>2</sub> groups of a lysine.

These lipid units may also be composed of, or contain, a residue of palmitic acid, 2-aminohexadecanoic acid, oleic acid, linoleic acid, linolenic acid, pimelautide, trimexautide, or a derivative of cholesterol, or any other natural lipid component of the cell membranes.

The lipopeptides constituting the mixed micelles or micro-aggregates are advantageously water-soluble in a proportion of at least 30% (by weight). These water-soluble lipopeptides have cationic surface-active properties, suitable for providing a solubilizing effect on other lipopeptides in weak acid medium.



The non-lipid part contains between 10 and 100, and preferably between 10 and 50 amino acids. The number of amino acids depends on the number of antigenic determinants constituting the non-lipid part of the lipopeptide and on their sizes, on the nature of the lipid part, and the proportions of the lipid and non-lipid parts.

The HTL and CTL antigenic determinants used are advantageously antigenic determinants able to bind with several different HLA, otherwise called multivalent or promiscuous antigenic determinants.

The HTL antigenic determinant used is preferably composed of the multivalent peptide 830-843 of the tetanus toxin.

QYIKANSKFIGITE

The glutamine (Q) of this sequence may optionally be acetylated.

Other multivalent HTL antigenic determinants may be the multivalent antigenic determinant of hemagglutinin (PREVOST-BLONDEL et al., 1995, J. Virol., vol. 62, n° 12, pages 8046-8055) or the PADRE antigenic determinant (ALEXANDER et al., 1994, Immunity, 1, 751).

The CTL antigenic determinant may be any antigenic determinant able to activate cytotoxic CD8<sup>+</sup> T lymphocytes.

It is preferably a CTL antigenic determinant of a protein presented by a tumour cell and in particular by a melanoma, a protein from HIV, from hepatitis B virus (HBV) or from papillomavirus, or protein p53.

It may in particular be one of the following antigenic determinants:

- antigenic determinants of protein BCR-ABL, resulting from the BCR-Abelson translocation (chronic myeloid leukemia) such as those listed in table

1.

- antigenic determinants of protein p53, such as those listed in table 2.

The antigenic determinants of protein p53 may in addition be comprised in the sequences 25-35, 63-73, 129-156, 149-156, 187-205, 187-234, 226-264, or 249-264 of this protein.

- antigenic determinants of proteins E<sub>6</sub> or E<sub>7</sub> of human papillomavirus (HPV), such as those listed in table 3.

- antigenic determinants of proteins of the HIV-1 virus such as those listed in table 4.

- antigenic determinants of melanoma or other tumours , such as those listed in tables 5, 6 and 7 and in particular antigenic determinants of the melan-  
5 A/mart-1 antigen of melanoma.

Other multivalent CTL antigenic determinants with a capacity to bind to class I HLA may be those included in the peptide 43-57 of HPV (GQAEPDRAHNIVTF) which contains HLA A2, A24, B7 and B18 antigenic determinants.

10 The CTL antigenic determinants may also be those of parasite antigens, and in particular of *Plasmodium falciparum*.

The mixed lipopeptide micro-aggregates or micelles according to the present invention may be freeze-dried, then taken up into any clinically acceptable buffer to be administered to the patients to be treated, and in  
15 particular to patients to be vaccinated.

They may be administered by any administration route used in therapeutics and, as non-limiting examples, by parenteral, percutaneous, oral, or sublingual routes or by intra-pulmonary nebulizer.

A further object of the present invention is thus the use of these  
20 lipopeptides for the production of a drug or vaccine for inducing a specific immune response, and in particular, for inducing an immune response against cancers such as melanoma, HIV and HBV viruses, papillomavirus, p53 or malaria.

Another object of the present invention is a pharmaceutical composition  
25 characterized in that it contains a pharmacologically active quantity of one or more of the lipopeptides described above, in addition to pharmaceutically compatible vehicles.

The present invention also relates to a method of inducing an immune  
30 response against an antigen comprising the administration of micelles or micro-aggregates, such as those described above, to an individual for which such a response is sought.

An additional object is a method of immunization against a pathogenic agent comprising the administration of micelles or micro-aggregates such as those described above to an individual for whom such an immunization is sought. Such pathogenic agents, and antigens, may be those listed above.

5       The lipopeptides forming the micelles according to the present invention may be produced by any suitable method known to a person skilled in the art. They may in particular be obtained by the Boc-benzyl or Fmoc-tert-butyl methods, in particular as disclosed in the application FR-90 15 870, which patent application is incorporated herein by reference.

10       The introduction of the lipid chain may be achieved in the solid phase, after selective deprotection of the functional group or groups concerned, as described in the article by DEPREZ et al., (1996, Vaccine, volume 14, n° 5, 375-382). The lipid chain may be introduced onto the  $\epsilon$ -NH<sub>2</sub> function of a lysine protected on the  $\alpha$ -NH<sub>2</sub> function by an F-moc group. The Fmoc-lys (Palm)  
15       obtained may then be used in solid-phase synthesis to produce the lipopeptide.

The micelles and micro-aggregates according to the present invention may be obtained by dispersing each lipopeptide in a concentrated acetic acid solution at about 80% concentration, then mixing the solutions thus obtained.

20       The quality of dissolution, i.e. the effective dispersion at the molecular level of each lipopeptide before the preparation of the mixture, is controlled by the two-dimensional nuclear magnetic resonance method (2DNMR). The resolution of the signal obtained during homonuclear experiments in two dimensions in a 600 MHz field confirms the complete dispersion, at the molecular level, of the lipopeptides in solution. The clarity of the mixture is not  
25       a sufficient criterion : in particular, the taking up of the lipopeptides by DMSO or a DMSO/water mixture does not lead, in most cases, to a sufficient dispersion state, which explains the ineffectiveness of the mixture studied by VITIELLO et al. (1995, cited above). Dissolution by acetic acid/water mixtures which are more dilute in acetic acid also does not lead in all cases to the  
30       preparation of a mixture of mixed micro-aggregates or micelles containing a statistical proportion of each constituent of the mixture at the microvolume level. In these two cases, even in the presence of an apparently clear mixture,

the sterilizing filtration over a 0.22  $\mu\text{m}$  membrane is either impossible, or irregular, with filtration yields which differ according to the constituents, which indicates that at the scale of a particle of this size, the representation of each constituent of the mixture has not been achieved. This micro-heterogeneity  
 5 compromises the immunogenicity of the mixture, since it compromises the simultaneous capture and presentation of all the constituents by a single antigen-presenting cell (APC), in the case of CTL and HTL antigenic determinants present on separate lipopeptides.

The present invention is illustrated, without in any way being limited, by  
 10 the following examples.

Figure 1 represents the chemical structure of the resin of type KNORR-MBHA.

Figures 2 and 3 show the two-dimensional nuclear magnetic resonance (2DNMR) spectra of a single lipopeptide (lipopeptide ENV) and a mixture of  
 15 lipopeptides respectively.

Figure 4 shows the helper response of eight macaques immunized with a mixture of lipopeptides.

Figures 5A to 5F show the cytotoxic response of macaque n° 109.

Figures 6A to 6D show the cytotoxic response of macaque n° 129.

20 Figures 7A and 7B show the cytotoxic response of macaque n° 127.

Figures 8, 9, 10 and 11 respectively show the cytotoxic responses of macaques n° 102, 105, 120 and 125.

Figures 12A, 12B and 12C respectively show the anti-N1, anti-G2 and anti-E cytotoxic activities of PBMC, CD8<sup>+</sup> and CD4<sup>+</sup> cells of the individual V4.1.

25 Figure 13 shows the cytolytic activity of PBMC of individual V4.5 collected twenty weeks after the beginning of immunization, stimulated in vitro with peptide N2 then tested for their CTL activity against wild vaccine (WT), or this same virus expressing a recombinant NEF protein (NEF, NEF-2, NEF-MN, NEF-A, NEF-ROD).

EXAMPLE 1 : Preparation of micelles or micro-aggregates according to the invention:

1- Description of lipopeptides used in the mixture

Name	Formula	Molecular weight
NEF 66	VGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLK(Pam)-NH <sub>2</sub>	3862.77
NEF 117	TQGYFPDWQNYTPGPGVRYPLTFGWICYKLVPK(Pam)-NH <sub>2</sub>	4017.754
NEF 182	EWRFDSRLAFHHVARELHPEYFKNK(Pam)-NH <sub>2</sub>	3451.04
GAG 183	DLNTMLNTVGGHQAAMQMLKETINEEAAEWDRK(Pam)-NH <sub>2</sub>	3983.65
GAG 253	NPPIPVGEIYKRWIILGLNKIVRMYSPTSILDK(Pam)-NH <sub>2</sub>	4063.05
ENV	TRPNNNTRKSIHIGPGRAFYTGEIIGDIRQAHK(Pam)-NH <sub>2</sub>	4027.69

5

CTL antigenic determinants represented :

RPNNNTRKSI	HLA-B27	TQGYFPDWQNY	HLA-B62
PPIPVGGEIY	HLA-B35	YFPDWQNYT	HLA-B17, B35
KRWIILGLNK	HLA-B27	TPGPGVRYPL	HLA-B7
LGLNKIVRMY	HLA-B62	RYPLTFGW	HLA-B27.2
QVPLRPMTYK	HLA-A3, A11, B27.2	YPLTFGWC	HLA-B18
VPLRPMTY	HLA-B35	AFHHVAREL	HLA-B52
AVDLSHFL	HLA-B62	FLKEKGGL	HLA-B8
AVDLSHFLK	HLA-A11		

15 This set of antigenic determinants shown above should lead to the induction of CTL responses in a large proportion of the human population, on the condition of being able to benefit from the helper effect of HTL antigenic determinants which, although not defined, are very probably present for simple statistical reasons in any one of the lipopeptides on condition however of

20 bringing together all the antigenic determinants, and thus all the peptide constituents of the mixture, in each micro-unit of volume.

2 - Synthesis

The solid phase approach was selected, using the Fmoc strategy for protecting the  $\alpha$ -amine function, and t-Bu for protecting the side chains. The

25 protocol used was a standard protocol based on the synthetic methods described by ATHERTON (Solid-phase synthesis, a practical approach, IRL

Press, 1989) and FIELDS and NOBLE (Int. J. Pept. Prot. Res., 1990, 35, 161-214).

The Fmoc-Lys(Palm)-OH was coupled to a resin of KNORR-MBHA type (figure 1). After deprotection of the alpha-amine function, the first amino acid was coupled (for example Fmoc-Leu-OH in the case of NEF 66). The coupling agent was TBTU (3 eq) in the presence of DIPEA (4.5 eq), with verification of coupling by a colorimetric test. A systematic acetylation was performed after a negative reaction had been obtained with this test, to minimize the risk of obtaining peptides by deletion. This succession of operations was repeated until all the amino acids in the sequence had been added.

After the synthesis, and deprotection of the terminal Fmoc group, the peptides were deprotected and cleaved by a TFA/water/DTT mixture (NEF 66, ENV), TFA/water/DTT/Ac-Trp-OH (GAG 183, GAG 253, NEF 117) or TFA/water/EDT/Ac-Trp-OH (NEF 182).

The peptides were each purified on a Vydac C18 column which was exclusively used for this purpose, at ambient temperature, with a water-acetonitrile solvent system, in perchlorate or TFA buffer.

They were then converted into their acetate form by ion exchange on a Dowex SBR column, then freeze-dried in 40% acetic acid.

Each peptide was produced from a single batch of synthesis and purification. No recycling of purification fractions was performed.

### 3 - Studies of the solubility of the lipopeptides:

#### 3-1) Use of pure water:

The peptides NEF 66, NEF 117, NEF 182 and ENV could be dissolved in pure water, at concentrations of up to 5 mg/ml. Peptide NEF 117 however gave a slightly opalescent solution. Peptides GAG 182 and GAG 253 were not soluble under these conditions.

The mixture of lipopeptides was however soluble in pure water, indicating that the hydrophilic lipopeptides were having a solubilizing effect on the less soluble peptides.

### 3-1) Use of DMSO:

The dissolution of lipopeptides is often performed using aqueous solutions of DMSO (dimethyl sulfoxide). This very powerful organic solvent is in fact compatible, after dilution, with the majority of biological tests carried out on cells or animals, even humans. The use of DMSO proved effective for good solution of peptides GAG 182 and GAG 253; the solutions obtained could then be diluted with water to reach a final concentration of 1 mg/ml in 20% DMSO/water; in these conditions, most of the peptides gave a clear solution, except GAG 183 for which a suspension was obtained.

It is useful to emphasize that even in the case of the clear solutions, and despite the compatibility of DMSO with Durapore filters, the solutions of lipopeptides in DMSO could not be filtered over filters of porosity 0.22  $\mu\text{m}$ , because they exerted a pressure incompatible with the mechanical resistance of the filters. This observation shows the formation of aggregates of size greater than 0.22  $\mu\text{m}$ . In some cases, we found it impossible to filter over filters resistant to solvents, with porosity 1  $\mu\text{m}$ , because of the formation of gels (this size of filter is in fact used to filter concentrated lipopeptide solutions before purification by RP-HPLC).

### 3-3) Use of 25% concentrated acetic acid:

The inclusion of the necessary step of sterilizing filtration thus requires the use of an organic solvent more suitable for dissociating the aggregates, compatible after dilution with freeze-drying, and non-toxic at low doses. Acetic acid was tested.

A minimum quantity of this solvent was initially used, defined as the quantity giving clear solutions at concentrations of 5 mg/ml : for peptides GAG 183 and GAG 253, 25% acetic acid was used; for the other peptides, dissolution in pure water was performed.

The solutions were subjected to nuclear magnetic resonance analysis in a 600 MHz field. It was observed that, despite the apparent clarity of the lipopeptide solutions of this series, even the most hydrophilic lipopeptides formed aggregates of significant size which prevented this type of study, in the absence of resolved signals.

Dissolution under these conditions did not lead to statistical dispersion, at the molecular level, of each of the constituents, despite the apparent clarity of the solutions.

#### 3-4) Use of 80% concentrated acetic acid:

The use of 80% concentrated acetic acid was then tested. The peptides were dissolved at a concentration of 1mM in 1 ml of 80% acetic acid (corresponding to : NEF 66 : 3.86 mg/ml; NEF 117 : 4.02 mg/ml; NEF 182 : 3.45 mg/ml; GAG 183 : 3.98 mg/ml; GAG 253 : 4.063 mg/ml; ENV : 4.027 mg/ml).

#### Analysis of the lipopeptides by proton NMR at 600 MHz

The lipopeptide samples were prepared by dissolving the lipopeptides in a solution of acetic acid (CD<sub>3</sub>COOD, 99.5% D atoms, EURISO-TOP, France)/H<sub>2</sub>O; 80:20 (V:V). 4 µl of a 50 mM solution of TMSP [sodium 3-(trimethylsilyl)propanesulfonate] in D<sub>2</sub>O were added as chemical shift reference. The final concentration of each peptide was 1 mM in at least 2 ml of solvent, which were transferred into 8 mm diameter NMR tubes (WILMAD 513A-7PP, Interchim, France).

The proton NMR spectra were performed on a BRUKER DMX600 NMR spectrometer fitted with an 8 mm BBI probe with z gradient, at a sample temperature of 310°K.

NOESY (Nuclear-Overhauser effect spectroscopy) experiments in two homonuclear dimensions according to Kumar et al. (1980, Biochem. Biophys. Res. Comm., 95, 1-6) and TOCSY (Total Correlation Spectroscopy) according to Bax and Davis (1985, J. Magn. Reson., 65, 355-360) and Griesinger et al. (1988, J. A. C. S., 110, 7870-7872) were obtained with 2048 x 512 complex points and processed after multiplication in two dimensions by a sine wave displaced by  $\pi/4$  with 2048 x 1024 points, for a spectral window of 12 ppm. The mixture times were 300 ms for the NOESY and 160 ms for the TOCSY. During the TOCSY mixture time, a MLEV 16 was applied with a B1 field of 7.8 KHz. So as to be under the same temperature conditions, the spin-lock time of the TOCSY was applied without resonance (+ or - 1 MHz) in the NOESY. The



suppression of water was achieved by using a slight pre-saturation of this signal during the relaxation time and the mixture time of the NOESY.

The high-field NMR analysis of the solutions showed perfectly resolved signals, allowing the TOCSY-NOESY experiments, and the complete sequential attribution of each lipopeptide. This result indicates complete dispersion, at the molecular level, of the lipopeptides in 80% acetic acid.

The 2D NMR spectrum of peptide ENV is shown on figure 2. The spectra of all the peptides could be obtained under the same conditions and interpreted. In order to verify if the intermixture of the solutions would change the dispersion of the lipopeptides, a 2D NMR spectrum of a virtual mixture was obtained by superimposing the 6 spectra obtained individually onto a single representation. It was compared with the 2D NMR spectrum actually obtained by mixing the solutions (figure 3). The resolution of the signals remained comparable, proving that none of the peptides had altered the solubility of the other constituents of the mixture. The sequence analysis required an accumulation of signals over 120 hours for each lipopeptide, during which period no significant alteration of the peptides was detected, either by NMR or by RP-HPLC. This observation thus allows the use of this solvent for the solubilization of the lipopeptides, their mixture, then filtration, even with a residence time of the order of 1 to 2 hours, conceivably necessary for the handling of relatively large volumes.

#### 4 - Studies of the sterilization filtration step:

##### 4 - 1 Isolated lipopeptides:

Tests on the sterilizing filtration were performed on 5 mg/ml solutions of each lipopeptide in water for peptides NEF 66, NEF 117, NEF 182 and ENV and in 25% acetic acid for peptides GAG 183 and GAG 253. The filtration yields for 1 ml over Millipore Millex GV SLGV 0130S filters (0.22  $\mu\text{m}$ ), followed by freeze-drying, are shown in table 8 (to within the precision of the determination).

These results are in agreement with the solution studies performed by NMR, and give information on the size of the aggregates or micelles detected: some peptides form aggregates of size greater than 0.22  $\mu\text{m}$ , and as a result

lead to mixtures containing micro-heterogeneities and an improbable simultaneous capture at the scale of the antigen-presenting cell.

#### 4 - 2) Preparation of different lipopeptide mixtures:

##### a) Preparation of batch CK2. Simple mixture of solutions, and production of a clear but micro-heterogeneous solution:

For the dissolution of the lipopeptides and the preparation of the mixture, solutions of totally clear appearance were intermixed, so as to evaluate the possible contribution of the surface-active character of lipopeptides ENV, NEF 66, NEF 117 and NEF 182. The conditions are summarized in table 9.

The solutions obtained were subjected to ultrasonic action to encourage the dispersion of the aggregates, mixed to give a final volume of 5.5.ml, and the mixture was again subjected to ultrasonic action, then diluted with 9.5 ml of water to obtain a final concentration of about 8% in acetic acid (AcOH), compatible with a good quality freeze-drying. This solution, after a final period in the ultrasonic bath, was filtered over Millipore Millex GV SLGV 0130S filters (0.22  $\mu$ m). The filtration yields for the peptides in the mixture were calculated for each lipopeptide, to give the results shown in the final column of table 9 (to within the precision of the determination).

The heterogeneity of the yields depending on the peptide showed the heterogeneity of the solution. Each peptide behaved as if it had been filtered individually : this behaviour was particularly evident for the peptide GAG 253, whose filtration yield from this solution in 8% acetic acid was lower than the yield observed when it was filtered alone from a solution of 25% acetic acid. This result confirms that, despite the apparent clarity of the mixture in dilute acetic solution, the mixture between the lipopeptides had not formed mixed micro-aggregates or micelles which contained in particular the more hydrophobic peptides. The exchanges of the lipopeptides between micelles occurs poorly under these conditions, and the surface-active function of lipopeptides ENV, NEF 66, NEF 117 and NEF 182 could not operate.

b) Preparation of batch CK3 : preparation of mixed micelles or micro-aggregates not including micro-heterogeneity

In order to guarantee complete mixing of the different lipopeptides at the level of each micro-unit of volume, a different strategy was followed :

5       - each lipopeptide was dissolved in 80% acetic acid so as to exploit the dissociating properties of this solvent.

      - in order to exploit the cationic surface-active properties expected of the peptides ENV, NEF 66, NEF 117 and NEF 182 in weak acid medium during the dilution step, the lipopeptides were dispersed in 80% acetic acid in the  
10 following order : 1 : ENV , 2 : NEF 66 , 3 : NEF 117 , 4; NEF 182, ending with the dispersion of the two most hydrophobic lipopeptides in a solution now concentrated in dissociating agents : acetic acid and cationic detergents. The fifth lipopeptide introduced was GAG 183 and the sixth GAG 253. An ultrasonic step was used at each stage to ensure effective dispersion of the  
15 aggregates.

      The solutions were mixed, then filtered over Millex GV SLGV 0130S filters (0.22  $\mu$ m). The filtration required a lower pressure than during the filtration of the 8% solution. The receiver vessels and the filter were then rinsed with water, in sufficient quantity to give a final acetic acid concentration  
20 of 8% (final volume 15 ml as before), so as to ensure the quality of the freeze-drying step. The filtration yields of the peptides in the mixture were calculated for each lipopeptide, to give the results listed in the final column of table 10 (to within the precision of the determination).

      The homogeneity of the yields confirms the homogeneity of the solution  
25 resulting from the dispersion at the molecular level at the time of filtration in concentrated acetic acid. The subsequent dilution cannot result in a reorganization of each peptide into monovalent entities, by application of the laws of entropy. This method of preparation of the mixture thus gives mixed micelles which each necessarily contain a statistical representation of each  
30 lipopeptide. The surface-active properties of the lipopeptides can operate and guarantee the solubility in water of vaccine doses after freeze-drying as well as the stability of the solutions during the handling time.

### c) Preparation of batch CK9

The procedure used was the same as for the previous batch, apart from the quantities. The solution of the peptide (20 mg/ml in 80% acetic acid) was filtered in 4 portions, changing the filter before its saturation, using identical  
 5 membranes (Durapore STERIVEX GV 0.22 µm sterile units (Millipore)), then made up with the water used for rinsing the filters and for dilution. The final volume was 1516 ml (including 154 ml of acetic acid : 10% in final solution). The portion volume was 1.3 ml per dose. The apportioned doses were freeze-dried, and analysed using a validated HPLC determination method. The  
 10 filtration yield for each lipopeptide is given below in table 11, and takes into account the determination sensitivity of each lipopeptide.

During the preparation of this batch, we again observed good homogeneity of the filtration yields, confirming the formation of mixed micelles or micro-aggregates, each micro-unit containing an equivalent proportion of  
 15 each constituent of the mixture.

The mixture after dilution and freeze-drying gave a white powder forming a compact homogeneous cake, which could very easily be taken up into solution in pure water or a solvent able to restore the osmolarity of the solution (5% glucose, 5% mannitol). The solution showed a very slight opalescence.  
 20 The pH obtained after taking up in a non-buffered solvent was 4.90. Raising the pH by 1 unit caused a slow precipitation: this behaviour contributed to the formation of a deposit during subcutaneous or intramuscular injection.

### d) Test of uniformity of concentration on batch CK9

According to the Pharmacopoeia, powders for parenteral use are  
 25 subjected to a requirement of uniformity of concentration. The test must be performed on 10 random samples, which are analysed individually for the active ingredient using an appropriate analytical method. The preparation satisfies the test if the concentration of each sample is between 85 and 115% of the average concentration.

30 The test was performed on 15 random samples, taken up in solution and diluted in 80% acetic acid according to a standardized operational procedure, so as always to inject an identical proportion of about 15 µg, defined during

preparation of the calibration curve. Each sample was injected three times, the concentration of each active ingredient corresponding to the average of the three values obtained.

The values obtained are given in table 12 below. The distribution of the values is clearly statistical. All the values are within a range defined for a two-sided test with  $P = 0.975$  (except for three deviant values, all from flask n° 1, which may correspond to a dilution error). The minimum and maximum values defined are within the limits imposed by the Pharmacopoeia (the difference observed was within 4 and 14.95% depending on the peptide, a variation linked to the inherent imprecision of the analysis method).

The absence of micro-heterogeneity of the solution was confirmed by the fact that the apportioned vaccine doses satisfied the concentration uniformity test.

#### EXAMPLE 2 - Preparation of a mixture of lipopeptides (SIV-Mortara 1) and test of immunogenicity in macaques.

##### 1) Preparation of the batch SIV-Mortara 1

This small batch was prepared in order to perform a pre-clinical test on macaques, to verify the tolerance and immunogenicity. This batch resulted from the mixture of the following lipopeptides :

Name	Formula
NEF 101	SVRPKVPLRAMTYKLAIDMSHFIEKK(Pam)-NH <sub>2</sub>
NEF 125	EKGGLGIIYYSARRHRILDMYLEK(Pam)-NH <sub>2</sub>
NEF 155	DWQDYTS GPGIRYPKTFGWLWKLVK(Pam)-NH <sub>2</sub>
NEF 201	SKWDDPWGEVLAWKFDPTLAYTYEAK(Pam)-NH <sub>2</sub>
NEF 221	YTYEAYARYPEELEASQACQRKRLEEGK(Pam)-NH <sub>2</sub>
GAG 165	KFGAEVVPGFQALSEGCTPYDINQMLNCVGDK(Pam)-NH <sub>2</sub>
GAG 246	QIQWMYRQQNPVGNIRRWIQLGLQKCVRMYNPTNK(Pam)-NH <sub>2</sub>
TT	Ac-QYIKANSKFIGITELKK(Pam)-NH <sub>2</sub>

Their mixing was performed from solutions in concentrated acetic acid, as for the preparation of batch CK3.

## 2) Immunogenicity in macaques.

### a) Materials and methods

The macaques, respectively numbered 102, 105, 109, 117, 120, 125, 127 and 129 were immunized by subcutaneous injection of the batch prepared  
5 above (500 µg), in sterile water, and were reinjected after periods of thirty days and sixty days.

These immunizations were performed in accordance with the directives of the European Union.

### Preparation of CTL lines

10 Blood cells (PBMC) were isolated by density gradient centrifugation through a lymphocyte separation medium (Pharmacia, Uppsala, Sweden). They were used immediately, or stored at -180°C in liquid nitrogen. Anti-peptide CTL lines were obtained by cultivating the monkey PBMC ( $2 \times 10^6$  cells/ml) in microtitration plates, in RPMI 1640 supplemented with penicillin  
15 (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), non-essential amino acids (1%), sodium pyruvate (1 mM), HEPES buffer (10 mM), 2-mercaptoethanol ( $2 \times 10^{-5}$  M) and 10% fetal calf serum (FCS) inactivated by heat.

The mixture of the seven free peptides, in other words without the lipid  
20 unit (5 µM of each), corresponding to the lipopeptide sequences was added to each well.

The plates were then incubated for three days at 37°C and interleukin-2 was added to each plate (10 IU/ml).

After seven days and fourteen days, the effector cells were stimulated by  
25 addition of new autologous PBMC, which had been in contact with the peptide mixture (5 µM of each) for two hours, then washed and irradiated (4000 rads).

### Determination of the proliferation of T cells

PBMC cells ( $2 \times 10^5$  in 200 µl per well) were cultivated in plates containing 1 µg/ml of lipopeptide TT (830-846), and 10 µg/ml of the peptide  
30 from tetanus toxin (TT).

After five days of culture, 1 µCi of [ $^3$ H]TdR was added to each well and the incubation was continued for eighteen hours. The cells were then collected

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using an automatic cell collector then the incorporation of tritiated thymidine was quantified using a scintillation counter.

#### Phenotypic analysis of CTL cell lines

The phenotype of the cell lines was determined the day that the chromium release assay was performed, by incubating the cells with anti-CD4 conjugated to FITC (OKT4, Ortho Diagnostic Systems, Raritan, NJ) and with anti-CD8 conjugated to phycoerythrin (Leu-2a, Becton Dickinson, Mountain View, CA) for thirty minutes at 4°C. The cells were washed with PBS buffer, then the percentage of coloured cells was determined using an Epics Elite flow cytometer (Coulter, Margency, France). Antibodies presenting a mixture of isotypes were used as controls.

#### In vitro conversion of B (B-LCL) cell lines

B (B-LCL) cell lines were obtained by incubating series dilutions of PBMC using the supernatant of cell line S 594. This line is infected by baboon herpes virus which immortalizes the cells (herpes virus papio). The B-LCL were then cultivated in the culture medium supplemented with 10% FCS.

#### Chromium release assay

The target cells were sensitized with the peptides.  $10^6$  B-LCL cells were incubated either overnight or for 1 hour, respectively, with the long or short peptides (concentration range  $10^{-5}$ M -  $10^{-8}$ M) at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. In order to obtain the target cells presenting the products of the SIVmac gene, the B-LCL were incubated at a concentration of  $10^6$  cells/ml with a recombinant vaccine virus (20 PFU/cell) for eighteen hours under the same conditions. The B-LCL were then washed and marked with 100 µCi Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (NEN Life Science Products, Courtaboeuf Les Ullis, France) for 1 hour, washed twice and used as target cells. The <sup>51</sup>Cr release was performed in microtitration plates. The cytolytic activity of the anti-SIV cell lines was measured by mixing  $5 \times 10^3$  target cells marked with chromium with the effector cells, at various ratios of effector cells to target cells, in a final volume of 200 µl/well. The plates were incubated for 4 hours at 37°C, then 100 µl of supernatant was taken from each well and analysed in a gamma radiation counter.

The spontaneous release of chromium was determined by incubating the target cells with medium alone. It never exceeded 20% of the total chromium incorporated.

The specific release of chromium was measured as follows:

$$100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm}).$$

The variation within a sample never exceeded 5%.

#### b) Results

Figure 4 shows the helper T response of the eight macaques.

Figures 5 to 11 show the cytotoxic response of the macaques.

The results of the immunizations with different peptides are summarized in table 13.

They show that seven of the eight macaques tested recognized different peptides, with macaques n° 109, 129 and 127 showing a particularly strong response.

The effectiveness of the induction of a CTL response confirms that the APC of the animals were able to capture and present one or more CTL antigenic determinants, and simultaneously the strong helper antigenic determinant present in the tetanus anatoxin and recognized by some of the animals.

#### EXAMPLE 3:

##### Preparation of a lipopeptide mixture (batch HG 1) for clinical tests in man.

A mixture of lipopeptides was defined for performing a clinical test (VAC 10), combining within the micelles the same peptide TT with sequences selected on the selection principle developed for VAC 04 (existence of one or more CTL antigenic determinants per sequence).

- the cysteine of peptide NEF 117 was replaced by a leucine : after synthesis and tests on cellular tests of several analogs of the CTL antigenic determinant nonapeptide containing this amino acid, it was observed that this replacement was possible; this modification avoided the stability problems due to the formation of a disulfide bridge.



- the peptide GAG 17 was selected from among other candidates for its strong cationic surface-active character, able to help to keep the other peptides in solution, and in particular GAG 253, which was retained in the mixture because of its immunogenicity in man.

- 5 The composition of this mixture, in which Pam represents a unit derived from palmitic acid and Ac the acetyl group, was the following :

Name	Formula
GAG 17	E K I R L R P G G K K K Y K L K H I V K(Pam)-NH <sub>2</sub>
GAG 253	N P P I P V G E I Y K R W I L G L N K I V R M Y S P T S I L D K(Pam)-NH <sub>2</sub>
POL 325	A I F Q S S M T K I L E P F R K Q N P D I V I Y Q Y M D D L Y K(Pam)-NH <sub>2</sub>
NEF 66	V G F P V T P Q V P L R P M T Y K A A V D L S H F L K E K G G L K(Pam)-NH <sub>2</sub>
NEF 116	H T Q G Y F P D W Q N Y T P G P G V R Y P L T F G W L Y K L K(Pam)-NH <sub>2</sub>
TT	Ac-Q Y I K A N S K F I G I T E L K K K(Pam)-NH <sub>2</sub>

- This set of peptides was synthesized as described in the previous examples. The mixture of solutions was performed on a sample of 5 mg of each peptide, dissolved at a concentration of 20 mg/ml in 80% acetic acid then  
 10 mixed in the following order : 1 : GAG 17; 2 : NEF 66; 3 : NEF 116; 4 : TT; 5 : GAG 253; 6 : POL 325.

- The yields from the operation of filtering the concentrated acetic acid solutions, followed by a dilution with water, proved comparable to the yields observed for the same operations with the mixture CK3 (to within the precision  
 15 of the determination). The homogeneity of the solubilities and the behaviour during the sterilizing filtration despite the heterogeneities of their individual chemical behaviour indicated the formation of mixed micelles.

**EXAMPLE 4:**

Preparation of a mixture of lipopeptides derived from antigen LSA3 for pre-clinical vaccination tests against the intrahepatic stage of *Plasmodium falciparum*, performed in mice and chimpanzees, then a clinical test in man.

Name	Formula
LSA3 CT1	LLSNIEEPKENIIDNLLNNIK(Pam)-NH <sub>2</sub>
LSA3 NRI	Ac-DELFNELLNSVDVNGEVKENILEESQK(Pam)-NH <sub>2</sub>
LSA3 NRII	Ac-LEESQVND DIFNSLVKSVQEQQHNVK(Pam)-NH <sub>2</sub>
LSA3 RE	K(Pam)VESVAPSVVEESVAPSVVEESVAENVVEESVAENV-NH <sub>2</sub>

- 5 This set of peptides was synthesized as described in example 1. The mixture of solutions was performed on a sample of 5 mg of each peptide previously dissolved at a concentration of 20 mg/ml in 80% acetic acid then mixed in the following order : 1 : LSA3 NRI; 2 : LSA3 NRII; 3 : LSA3 CT1; 4 : LSA3 RE. The yields from the operation of filtering the concentrated acetic acid solutions, followed by a dilution with water, proved comparable for all the lipopeptides.

**EXAMPLE 5:**

Study of the immune response in man after injection of micelles from batch CK9.

- 15 1. Materials and methods

Micelles used:

The micelles which were injected were obtained as described in example 1 for batch CK9.

Long and short peptides.

- 20 The following long peptides corresponding to the immunogenic lipopeptides were synthesized (the positions of the amino acids on proteins NEF, GAG and ENV are given in parentheses) : N1 (NEF 66 to 97), N2 (NEF 117 to 147), N3 (NEF 182 to 205), G1 (GAG 183 to 214), G2 (GAG 253 to 284) and E (ENV 303 to 335).

- 25 The following short peptides, including the lipopeptide sequences already known to be the minimal CTL antigenic determinants, were synthesized by Neosystem (Strasbourg, France):

NEF 121-128, NEF 137-145, NEF 184-191 and NEF 195-202 restricted to HLA-A1.

NEF 136-145, NEF 190-198 and GAG 183-191 restricted to HLA-A2.

NEF 73-82, NEF 84-92 and EBN 416-424 HLA restricted to HLA-A11.

5 NEF 90-97 and NEF 182-189 restricted to HLA-B8.

NEF 134-141 and GAG 263-272 restricted to HLA-B27.

NEF 135-143 restricted to HLA-B18.

Immunization protocol:

Volunteers were immunized by subcutaneous injection of the micelles, or  
10 the six corresponding lipopeptides, in the presence of QS21 adjuvant. The lipopeptides or micelles were injected in different ways depending on the individual.

Volunteers V4.6, V4.15, V4.16, V4.17, V4.18, and V4.28 were immunized with the six lipopeptides in the form of micelles.

15 Volunteer V4.6 received 250 µg of each of the lipopeptides.

Volunteers V4.15, V4.16, V4.17, V4.18, and V4.28 were immunized with 500 µg of each of the six lipopeptides.

Volunteers V4.5, V4.1, V4.19, V4.21, V4.32 and V4.34 were immunized with the six lipopeptides in the presence of QS21 adjuvant.

20 Volunteer V4.5 received 100 µg of the 6 lipopeptides, while volunteers V4.1, V4.19, V4.21, V4.32 and V4.34 each received 500 µg of the six lipopeptides.

All the volunteers were immunized three times with the mixture of the six lipopeptides, the two later injections being performed 4 weeks and 16 weeks  
25 respectively after the first injection.

Blood samples were taken after the first injection (hereafter referred to as week 0) and 20 weeks after the first injection (week 20).

Peripheral blood mononuclear cells (PBMC) and serum were isolated by conventional methods, and frozen.

ELISA detection of HIV anti-peptide antibodies of immunoglobulin G (IgG) type.

Wells of polystyrene plates were covered with 5 µg/ml of the peptides (N1, N2, N3, G1, G2 or E) overnight at 4°C. Saturation was performed using a  
 5 PBS solution containing 0,1% Tween 20 and 3% bovine serum albumin (BSA). Diluted serums (1/100) were incubated in the covered wells overnight at 4°C and the bound antibodies were detected using goat anti-human IgG conjugated with alkaline phosphatase (1/5000, Sigma). The phosphatase activity was measured using 4-methyl umbelliferyl phosphate as substrate (Sigma), and the  
 10 fluorescence measurement was performed at 360/460 nm in a Cytofluor 2300 (Millipore).

Measurement of the T cell responses directed against HIV peptides.

PBMC ( $10^5$  per well) were cultivated in complete medium with 1 µg/ml or 0.2 µg/ml of the soluble peptides (N1, N2, N3, G1, G2 or E). The proliferation  
 15 was determined after 5 days culture by added 1 µCi/well of tritiated thymidine (NEN, Paris) 12 hours before their collection.

The capacity of the PBMC to proliferate in vitro was verified using independent cultures performed over 5 days with phytohemagglutinin A (PHA) of PPD (Tuberculin purified derivative Reference Statens Serumins Institute  
 20 n° 2390), tetanus toxin (TT) and SEB (enterotoxin B of Staphylococcus golden, Reference Sigma S4881), at 1 µg/ml and 10 µg/ml respectively.

Removal of the CD4<sup>+</sup> and CD8<sup>+</sup> T cells of the PBMC was performed with anti-mouse immunoglobulins and by complement activation. To summarize,  $10^7$  PBMC were incubated in 1 ml of medium lacking bovine serum albumin for  
 25 30 minutes at 4°C with 2 µg of monoclonal antibody OKT4 or OKT8 (Ortho Diagnostic Systems).

1 ml of diluted rabbit serum complement (Hoechst Behring, Reuil, France) was added over 45 minutes at 37°C. The cell suspension was washed twice so as to remove the unbound complement. The resuspended cells were  
 30 analysed using flow cytometry. Analysis of the phenotypes using anti-CD4<sup>+</sup> and anti-CD8<sup>+</sup> antibodies was performed to verify the enrichment. Finally, the

cells resulting from the removal of the CD4<sup>+</sup> and CD8<sup>+</sup> cells were tested in a proliferation test.

#### Preparation of CTL cell lines

In vitro stimulation of the PBMC was performed by mixing 10<sup>6</sup> PBMC (responsive cells) with 10<sup>6</sup> irradiated stimulant cells (autologous PBMC incubated for 2 hours with different peptides) in complete RPMI culture medium (RPMI 1640 supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, nonessential amino acids and 10% heat-inactivated bovine serum albumin).

10 U/ml of interleukin -2 were added after 3 days. The responsive cells were restimulated each week for 3 or 4 weeks using peptides incubated with autologous PBMC (prepared in the same way as on day 0), in a medium supplemented with 10 U/ml of interleukin-2. After 3 or 4 stimulations, the CTL cells were tested using the EBV autologous cell line as target overnight with 10 µg of the different peptides (N1, N2, N3, G1, G2 or E) for 10<sup>6</sup> cells.

In order to obtain the target cells presenting the products of the HIV gene, EBV target cells were infected, at a rate of 10<sup>6</sup> cells/ml, with a wild type (WT) vaccine virus or with HIV-1/LAI, HIV-1/MN, HIV/A or HIV/ROD NEF recombinant vaccine viruses overnight (20 PFU/cell).

The different target cells were then washed and marked with 100 µCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (NEN Life Science Products, Les Ullis, France).

The cytolytic activity was measured in a <sup>51</sup>Cr release test, over 4 hours. The average spontaneous release did not exceed 20% of the total <sup>51</sup>Cr incorporation.

The results are expressed as follows :

specific release of chromium = 100 x (measured cpm/spontaneous cpm)/(maximum cpm - spontaneous cpm).

The removal of the CD4<sup>+</sup> and CD8<sup>+</sup> cells from the PBMC was performed as described above.

#### ELISPOT γ-interferon test

96-Well microcells plates (MultiScreen-HA, Millipore S.A., Molsheim, France) were covered with 5 µg/ml of mouse anti-human-γ-interferon antibody,

as capture antibody (Genzyme Corporation, Cambridge, Massachusetts, USA) overnight at 4°C.

After washing, the wells were saturated with complete RPMI medium and PBMC which had been freshly isolated, or kept cold, were added (2 x 10<sup>5</sup> cells per well) with different peptides corresponding to the minimal CD8<sup>+</sup> antigenic determinants (10 µg/ml).

After 24 hours incubation at 37°C in an incubator (5% CO<sub>2</sub>), the plates were washed and incubated for 2 hours with 100 µl rabbit anti-human-γ-interferon polyclonal antibody (1/250, Genzyme). After washing, a rabbit anti-IgG-biotin conjugate (1/500, Boehringer Mannheim France S.A., Meylan, France) was incubated for 1 hour. Finally, extravidine marked with alkaline phosphatase (Sigma-Aldrich Chimie S.A.R.L., St Quentin Fallavier, France) was added over 1 hour.

100 µl of alkaline phosphatase chromogenic substrate (Bio Rad Laboratories, Hercules, CA, USA) were added to develop the spots. The blue spots were then counted using a microscope.

The negative control consisted of PBMC incubated alone in the medium, or incubated with a peptide corresponding to a CD8<sup>+</sup> antigenic determinant derived from the HIV virus presented by adapted HLA.

The positive control consisted of activating the PBMC with 50 mg/ml of PMA (Phorbol myristate acetate, reference Sigma P 8139) and 500 ng/ml of ionomycin (100 to 300 PBMC per well were added).

This strong mitogen stimulation allowed measurement of the viability of the T lymphocytes, and verification of the quality of the storage in the cold.

## 2. Results

### Tolerance of the treatment.

The secondary effects from the injection of the lipopeptides were not serious. An epidermal reaction was observed at the injection site. Local reactions consisted of small erythemas lasting only 24 to 48 hours. These effects were in no case associated with systemic symptoms. These observations show that the lipopeptides are well tolerated in normal individuals.

### Specific induction of a humoral response against HIV-1 peptides

Serum samples were collected before the start of the vaccinations (week 0) and at the twentieth week, after the third injection.

The serums from the immunized volunteers were tested by ELISA for the presence of IgG antibody directed against the NEF (N1, N2, N3), GAG (G1, G2), and ENV (E) peptides

No IgG specific for the HIV peptides was detected before the injection in the twelve subjects listed in table 14.

At the twentieth week, anti-N1 IgG antibodies were detected in five of the vaccinated subjects (V4.6, V4.28, V4.1 (SQ21), V4.32 (QS21), and V4.34 (QS21)), and anti-N2 IgG antibodies were detected in the serums of ten of the subjects, among the twelve vaccinated. No antibody of type anti-N3 IgG was detected. The titration in anti-N2 antibody was negative in the serums of individuals V4.17 and V4.18. The antibody titration was three to five times greater than that of the negative control in the serums of V4.15, V4.16, V4.1 (QS21), V4.5 (QS21) and V4.21 (QS21). The antibody titration was five to ten times greater than that of the negative control in the serums of V4.6, V4.19 (QS21), and V4.28. Finally, the serums of patients V4.32 (QS21) and V4.34 (QS21) showed antibody titration at least 10 times greater than that of the negative control.

After 3 injections, no anti-G1 IgG antibody was detected, but anti-G2 IgG antibodies were detected in the serums of the 12 individuals vaccinated. The anti-G2 antibody titration was 2 to 3 times greater than that of the negative control for patient V4.18 (QS21), the antibody titration was 5 to 10 times greater than that of the negative control for individuals V4.16, V4.17, V4.5 (QS21), V4.19 (QS21) and V4.21 (QS21). The serums of patients V4.6, V4.15, V4.28, V4.1 (QS21), V4.32 (QS21) and V4.34 (QS21) had an antibody titration more than ten times greater than that of the negative control. The serums of 6 of the 12 individuals tested, V4.28, V4.1 (QS21), V4.5 (QS21), V4.19 (QS21), V4.32 (QS21) and V4.34 (QS21) contained specific anti-E antibodies.

Specific helper T cell response of the HIV-1 virus peptides.

The proliferative responses with respect to the soluble peptides obtained with the PBMC cells of the different individuals vaccinated are shown in table 15.

5        The NEF, GAG and ENV peptides caused proliferation of the donor PBMC only, after the vaccination. The PBMC of the individuals immunized with the lipopeptides (with or without QS21 adjuvant) proliferated against at least one peptide after 20 weeks (4 weeks after the third injection, for 8 subjects out of 10 given in table 15).

10        No proliferation was observed for the PBMC of individuals V4.15 and V4.17.

15        The PBMC of individual V4.6 proliferated in response to peptides N3, G1 and G2 with a proliferation index of between 4 and 10. An induction of the proliferation in response to G1, G2 and E was observed with the PBMC of individual V4.16. The PBMC of individual V4.28 were able to proliferate in response to peptides N1, N3, G2 and E. A proliferative response against peptides N1, G2 and E was observed for the PBMC of vaccinated individual V4.5.

20        A strong proliferation was observed in response to peptides N1, G2 and E with the PBMC of individual V4.19 (QS21), which in addition were able to proliferate in the presence of N2 and N3.

The PBMC of individual V4.21 proliferated in the presence of N1 and G2, while those of individuals V4.32 proliferated only in the presence of G2.

25        A proliferative response was observed in the presence of N1, N3, G2 and E with the PBMC of individual V4.34 (QS21).

30        Overall the third immunization with the lipopeptides induced a proliferative response against peptide N1 for five of the ten subjects treated, against N2 for one of the ten subjects treated, N3 for four of the ten subjects treated, G1 for two of the ten subjects treated, G2 for eight of the ten subjects treated, and finally E for five of the ten subjects treated.



The removal experiments performed with the PBMC from the different individuals vaccinated showed that the proliferation of the PBMC recovered after twenty weeks occurred preferentially via the helper CD4<sup>+</sup> T cells.

#### Induction of CTL activity specific to HIV

5 The PBMC obtained before and after the immunizations were stimulated in vitro and tested for their CTL activity specific to HIV.

The results of representative experiments are given in table 16.

The specific CTL activity was tested against the EBV autologous cell line, incubated with or without the NEF, GAG and ENV peptides. No anti-HIV  
10 response was detected with the PBMC recovered before the immunization. A specific CTL activity was detected in the PBMC collected three weeks after the immunization for nine of the twelve individuals.

Table 16 summarizes the cytotoxic activity of eight of the vaccinated individuals, the activity of one other individual being shown in figure 12.

15 At least one peptide contained in the lipopeptide vaccine induced specific CTL effector cells recognizing the HIV peptides.

For example, the PBMC of individual V4.6 recognized in a cytotoxic test the EBV autologous cells stimulated with peptides G2 and E. The PBMC of individual V4.16 recognized peptide N3 and E. The percentage of lysis was  
20 variable, weak for individual V4.16 recognizing peptide N3, intermediate for individual V4.18 with peptide N1 and strong for individual V4.5 (QS21) with peptides N2 and G2.

A specific CTL activity was also generated against peptides containing a minimal CD8<sup>+</sup> HIV antigenic determinant (individuals V4.16 and V4.28).

25 In order to evaluate whether the effector cells are CD8<sup>+</sup> T cells, as might be expected for the CTL specific for class-I restricted antigens, the CD8<sup>+</sup> or CD4<sup>+</sup> T lymphocytes were removed from the PBC and a cytotoxicity test was performed.

In representative experiments performed with the PBMC of individual  
30 V4.1 (figure 12), an effective lysis was observed of the autologous EBV cells incubated with the HIV peptides, for the PBMC and the CD8<sup>+</sup> cells. Enrichment

in CD8<sup>+</sup> cells increased the percentage of specific lysis. These results confirm that the anti-HIV cytotoxic activity operates via CD8<sup>+</sup> T cells.

It was also important to determine whether the cytotoxic T cells (CTL) recognized and lysed cells infected with the virus.

5 PBMC from individual V4.5, collected 20 weeks after immunization, and stimulated in vitro with peptide N2, were thus tested for their CTL activity against autologous targets infected with different viruses expressing the recombinant NEF protein. The results of representative experiments are given in figure 13. Anti-peptide N2 CTL obtained from individual V4.5 (QS21)  
10 recognized an antigen naturally modified by autologous EBV-LCL infected with recombinant viruses of the vaccine coding for the HIV-NEF genes obtained from different strains of HIV.

For the same effector/target ratio, CTL specific to the HIV virus recognized NEF-LAI and NEF-MN with the same effectiveness. A lower  
15 percentage of specific lysis was obtained for the NEF-A protein or the NEF-ROD protein.

These results show that the CTL obtained after vaccination with the lipopeptides are able to recognize different strains of the HIV virus.

#### CD8<sup>+</sup> T cells secreting $\gamma$ -interferon ex-vivo, specific to the HIV virus

20 Effector CD8<sup>+</sup> T cells may exercise a lytic activity and/or produce lymphokines. The quantity of CD8<sup>+</sup> T cells secreting  $\gamma$ -interferon was thus evaluated by a specific ELISPOT test.

A recent study has shown that the intracellular inactivation of the hepatitis B virus occurs via CD8<sup>+</sup> T cells secreting specific and cytotoxic  $\gamma$ -  
25 interferon, which induced a protective immunity. This approach was used to identify the minimal antigenic determinants of CD8<sup>+</sup> T cells by sensitization of PBMC with the short peptides. All the short peptides used have already been described as being CTL antigenic determinants (see above).

An ELISPOT has also been used to quantify ex vivo the number of CD8<sup>+</sup>  
30 T cells secreting  $\gamma$ -interferon specific to HIV peptides in the PBMC of the vaccinated subjects (table 17).

### Conclusion

This study has shown that a lipopeptide vaccine in the form of micelles, and without adjuvant, containing different HIV antigenic determinants contained in the viral proteins NEF, GAG and ENV of the HIV virus, is able to induce a strong and persistent multi-antigenic determinant B and T response in man.

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**TABLE 1: Antigenic determinants of BCR-ABL**

Peptide	Sequence	Fixation to HLA
247-255	EDAE LNPRF	B44
488-496	SELDLEKGL	B44
768-776	DELEAVPNI	B44
901-934	b2a2 KEDALQRPV	B44
902-935	b2a2 EDALQRPVA	B44
986-994	GEKLRVLGY	B44
1176-1184	EDTMEVEEF	B44
1252-1260	MEYLEKKNF	B44
1691-1699	NEEA ADEVF	B44
49-57	VNQERFRMI	B 8
580-588	LFQKLASQL	B 8
722-730	ARKLRHVFL	B 8
786-794	ALKIKISQI	B 8
886-893	CVKLQTVH	B 8
928-936	b3a2 KALQRPVAS	B 8
1830-1838	GA KTKATSL	B 8
1975-1983	IQQMRNKFA	B 8
1977-1984	QMRNKFAF	B 8
252-260	NPRFLKDNL	B7
329-338	TPDCSSNENL	B7
693-701	TPRRQSMTV	B7
1058-1066	SPGQRSISL	B7
1196-1205	HPNLVQLLG V	B7
1560-1569	SPKPSNGAGV	B7
1717-1725	KPLRRQVTV	B7
1878-1884	SPAPVPSTL	B7
36-44	ERCKASIRR	B27
71-79	DRQRWGFFRR	B27
575-583	QRVGDLFQK	B27

**TABLE 1: Antigenic determinants OF BCR-ABL**

Peptide	Sequence	Fixation to HLA
834-842	FRVHSRNGK	B27
642-650	LLYKPVD RV	A2
684-692	FLSSINEEI	A2
708-716	QLLKDSFMV	A2
714-722	FMVELVEGA	A2
817-825	KLSEQESLL	A2
881-889	MLTNSCVKL	A2
908-917	GLYGFLNVIV	A2
912-920	FLNVTVHSA	A2
1240-1248	VLLYMATQI	A2
1903-1911	FIPLISTRV	A2
1932-1940	VVLDSTEAL	A2
50-58	NQERFRMIY	A1
223-231	VG DASRPPY	A1
549-558	KVPELYEIHK	A3/A11
583-591	KLASQLGVY	A3/A11
715-724	MVELVEGARK	A3/A11
916-923	IVHSATGFK	A3/A11
920-928	b3a2 ATGFKQSSK	A3/A11
924-932	b3a2 KOSSKALQR	A3/A11
1156-1165	EVYEGVWKKY	A3/A11
1311-1320	SLAYNKFSIK	A3/A11
1499-1509	NLFSALIKK	A3/A11
1724-1734	TVAPASGLPHK	A3/A11
1905-1914	LISTRVSLRK	A3/A11
1922-1930	RIASGAITK	A3/A11
924-936 b3a2	KOSSKALQRPVAS	DR4

TABLE 2 - Antigenic determinants of p53

- antigenic determinants of p53 binding to HLA-A1:

RVEGNLARVEY (196-205)

GSDCTTIHY (226-234)

- antigenic determinants of p53 binding to HLA-A2:

LLPENNVLSPL (25-35)

RMPEAAPPV (65-73)

RMPEAAPRV

ALNKMFCQL (129-137)

STPPPGTRV (149-157)

GLAPPQHLIRV (187-197)

LLGRNSFEV (264-272)

PLDGEYFTL (322-330)

- antigenic determinants of p53 binding to HLA-A3:

RVRAMAIYK (156-164)

RRTEENLR (282-290)

ELPPGSTKR (298-306)

- antigenic determinants of p53 binding to HLA-B7:

LPENNVLSPL (26-35)

APRMPEAAPPV (63-73)

APRMPEAAPRV

APPQHLIRV (189-197)

RPILTIITL (249-257)

KPLDGETYFTL (321-330)

- antigenic determinants of p53 binding to HLA-B8:

CQLAKTCPV (135-143)

GLAPPQHLLI (187-195)

NTFRHSVVVV (210-218)

- antigenic determinants of p53 binding to HLA-B51:

LLPENNVLSPL (25-35)

RMPEAAPPV (65-73)

LIRVEGNLRV (194-203)

TABLE 3Antigenic determinants of proteins E<sub>6</sub> and E<sub>7</sub>

YMLDLQPETT (E7 11-20)  
LLMGTLGIV (E7 82-90)  
TLGIVCPI (E7 86-93)  
TIHDIILECV (E6 29-38)  
KLPQLCTEL (E6 18-26)  
RPPKLPQL (E6 8-15)  
LRREVYDFAFRDLCIVYRDGNPY (E6 45-67)  
ISEYRHICY (E6 80-88)  
EKQRHLDKKQRFHNIRGRWT (E6 121-140)  
GQAEPDRAHYNIVTF (E7 43-57)  
QAEPDRAHY (E7 44-52)  
EPDRAHYNIV (E7 46-55)

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HLA-A1

HLA-A2

Gp120 121-129: KLTPLCVTL  
P17 77-85: SLYNTVATL  
RT 200-208: ALVEICTEM  
RT 275-285: VLDVGDAYFSV  
RT 346-354: KIYQYMDDL  
RT 368-376: KIEELRQHL  
RT 376-387: LLRWGLTTPDK  
RT 476-484: ILKEPVHGV  
RT 588-596: PLVKLWYQL  
RT 683-692: ELVNQIIEL  
Nef 136-145: PLTFGWCFKL  
Nef 180-189: VLOWRWFDSRL  
Nef 190-198: ALHHVAREL  
Gp41 818-826: SLLNATVDI  
P24 185-193: DLNTMLNTV  
RT 346-354: VIYQYMDDL  
RT 588-596: PLVKLWYQL  
Pro 143-152: VLVGPTPVNI  
(Gp120 37-44: TVYYGVPV  
(Gp120 115-122: SLKPCVKL  
(Gp120 313-321: RIQRGPGRA  
(Gp120 197-205: TLTSCNTSV  
(Gp120 428-435: FINMWQEV  
(Gp 41 836-844: VVQGAYRAI  
(p24 219-228: HAGPIAPGQM  
(p15 422-431: QMKDCTERQA  
(p15 448-456: FLQSRPETA  
(RT 681-691: ESELVNQIEG



TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-A3

P17 18-26: KIRLRPGGK  
 P17 20-28: RLRPGGKKK  
 RT 200-210: ALVEICTEMEK  
 RT 325-333: AIFQSSMTK  
 RT 359-368: DLEIGQHRTK  
 Nef 73-82: QVPLRPMTYK  
 Gp120 37-46: TVYYGVPVWK  
 Gp41 775-785: RLRDLLLLIVTR  
 P17 18-26: KIRLRPGGK

HLA-A11

RT 325-333: AIFQSSMTK  
 RT 507-517: QIYQEPFKNLK  
 Nef 73-82: QVPLRPMTYK  
 Nef 84-92: AVDLSHFLK  
 p24 349-359: ACQVGGPGHK  
 P17 83-91: ATLYCVHQR

HLA-A24 (A9)

Gp120 52-61: LFCASDAKAY  
 Gp41 591-598: YLKDQQLL  
 or 590-597 RYLKDQQLL  
 (RT 484-492: VYYDPSKDL  
 (RT 508-516: IYQEPFKNL  
 (RT 681-691: ESELVNQIIEG

HLA-A25 (A10)

P24 203-212: ETINEEAAEW

HLA-A26 (A10)

P24 167-175: EVIPMFSAI

HLA-A30 (A19)

(Gp41 845-852: RAIRHIPRR

HLA-A31 (A19)

(Gp41 775-785: RLRDLLLLIVTR

HLA-A32 (A19)

Gp120 424-432: RIKQIINMW  
 (Gp41 774-785: HRLRDLLLLI  
 RT 559-568: PIQKETWETW

TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-A33 (A19)

(P24 266-275 :IILGLNKIVR

HLA-B7

RT 699-707: YLAWVPAHK  
 Nef 68-77: FPVTQVPLR  
 Nef 128-137: TPGPGVRYPL  
 Gp120 303-312: RPNNNTRKSI  
 Gp41 848-856: IPRRIRQGL  
 RT 699-707: YLAWVPAHK

HLA-B8

Gp120 2-10: RVKEKYQHL  
 P17 24-32 :GGKKKYKLL  
 Nef 90-97: FLKEKGGL  
 P24 259-267: GEIYKRWII  
 Gp41 591-598: YLKDQQLL  
 (Gp41 849-856: PRRIRQGL  
 or 851-859: RIRQGLERIL  
 (P24 329-337: DCKTILKAL  
 (RT 185-193: GPKVKQWPL  
 (Nef 182-189: EWRFD SRL

HLA-B14

Gp41 589-597: ERYLKDQQL  
 P24 298-306: DRFYKTLRA  
 (P24 183-191 ? : DLNTMLNTV  
 (p24 304-313: LRAEQASVQEV  
 (p24 305-313: RAEQASVQEV

HLA-B18

(Nef 135-143: YPLTFGW CY  
 (Nef 135-143: YPLTFGW CF

**TABLE 4: Antigenic determinants of the HIV-1 virus (continued)**

HLA-B27

P24 263-272: KRWIHLGLNK  
Nef 73-82: QVPLRPMTYK  
Nef 134-141: RYPLTFGW  
or 133-141 : YPLTFGW  
Gp41 589-597 ERYLKDQQL  
(Gp41 791-800: GRRGWEALKY

HLA-B35

Gp120 78-86: DPNPQEVVL  
Gp120 257-265: RPVVSTQLL  
RT 285-294: VPLDKDFRKY  
RT 323-331: SPAIFQSSM  
RT 342-350: NPDIVTYQY (consensus clade B)  
RT 460-468: IPLTEEAEL  
RT 598-608: EPIVGAETFY  
Nef 68-76: FPVRRPQVPL  
Nef 74-81: VPLRPMTY  
Gp41 611-619: TAVPWNASW  
Gp120 42-52: VPVWKEATTTL  
P17 124-132: NSSQVSQNY (consensus clade B)  
P24 254-262: PPIPVGEIY(consensus clade B)

HLA-B37

Nef 120-128: YFPDWQNYT

HLA-B44 (B12)

P24 178-186: SEGATPQDL  
(p24 175-184: LESGATPQDL

TABLE 4: Antigenic determinants of the HIV-1 virus (continued)

HLA-B51 (B5)

gp41 562-570: RAIEAQQHL  
 RT 200-208: ALVEICTEM  
 RT 209-217: EKEGKISKI  
 RT 295-302: TAFTIPSI

HLA-B52 (B5)

Nef 190-198: AFHHVAREL

HLA-B55 (B22)

Gp120 42-51: VPVWKEATTTL

HLA-B57 and B58 (B17)

P24 240-249: TSLTQEQIGW  
 Nef 116-125: HTQGYFPDWQ  
 or 116-124: HTQGYFPDW  
 Nef 120-128: YFPDWQN  
 (P24 147-155: ISPRTLNAW  
 (P24 164-172: FSPEVIPMF

HLA-Bw62 (B15)

P17 20-29: RLRPGGKKKY  
 P24 268-277: LGLNKIVRMY  
 RT 427-438: LVGKLNWASQIY  
 Nef 84-91: AVDLSHFL  
 Nef 117-127: TQGYFPDWQNY

HLA-Cw4

gp120 380-388: SFNCGGEFF

HLA-Cw8

RT 663-672: VTDSQYALGI  
 P24 305-313: RAEQASQEV  
 Nef 82-91: KAALDLSHPL

HLA-Cw?

P24 308-316: QATQEVKNW

**TABLE 5 - Antigenic determinants of human melanoma**

Gene/protein	MHC restriction	Peptide	Amino acid positions
Tyrosinase	HLA-A2	MLLAVLYCL	1-9
	HLA-A2	YMNGTMSQV	369-377
		YMDGTMSQV	
	HLA-A24	AFLPWHRLF	206-214
	HLA-B44	SEIWRDIDF	192-200
	HLA-DR4	QNILLSNAPLGPQFP	56-70
		SYLQDSDPDSFQD	450-462
Pmel17 <sup>gp100</sup>	HLA-A2	KTWGQYWQV	154-162
	HLA-A2	AMLGHTTMEV	177-186
	HLA-A2	MLGHTTMEV	178-186
	HLA-A2	ITDQVPFSV	209-217
	HLA-A2	YLEPGPVTA	280-288
	HLA-A2	LLDGTATLRL	457-466
	HLA-A2	VLYRYGSFSV	476-485
	HLA-A2	SLADTNSLAV	570-579
	HLA-A3	ALLAVGATK	17-25
	HLA-A2	(E)AAGIGILTV	26(7)-35
Melan-A <sup>MART-1</sup>	HLA-A2	ILTVILGVL	32-40
GP <sup>75TRP-1</sup>	HLA-A31	MSLQRQFLR	
TRP-2	HLA-A31	LLGPGRPYR	197-205

**TABLE 6: Tumour Antigenic determinants resulting from de mutations**

Gene/protein	Tumour	MHC restriction	Peptide	Amino acid positions
MUM-1	Melanoma	HLA-B44	EEKLIVVLF	30-38
CDK4	Melanoma	HLA-A2	ACDPHSGHFV	23-32
$\beta$ -catenine	Melanoma	HLA-A24	SYLDSGIHF	29-37
HLA-A2	Renal carcinoma			-
CASP-8	Squamous carcinoma of head and neck	HLA-B35	FPSDSWCYF	476-484

**TABLE 7****Antigens common to different tumours**

Gene	Normal expression tissue	MHC restriction	Antigenic peptide	Amino acid positions
MAGE-1	testicles	HLA-A1 HLA-Cw16	EADPTGHSY SAYGEPRKL	161-169 230-238
MAGE-3	testicles	HLA-A1 HLA-A2 HLA-B44	EVDPIGHLY FLWGPRALV MEVDPIGHLY	168-176 271-279 167-176
BAGE	testicles	HLA-Cw16	AARAVFLAL	2-10
GAGE-1/2	testicles	HLA-Cw6	YRPRPRRY	9-16
RAGE-1	retina	HLA-B7	SPSSNRIRNT	11-20
GnTV	none	HLA-A2	VLPDVFIRC	38-64
mucin	breasts during lactation	no restriction	PDTRPAPGSTAPPA HGV TSA*	

\* Aberrant transcript of the N-acetyl glucosaminyl transferase V (GnTV) found only in melanomas

**TABLE 8**

LIPOPEPTIDES	FILTRATION YIELD
NEF 66	quantitative
NEF 117	80%
NEF 182	quantitative
GAG 183	80%
GAG 253	77%
ENV	quantitative

**TABLE 9**

Peptide	Solvent	Concentration (mg/ml)	Volume removed (ml)	Filtration yield (%) after mixing
NEF 66	water	5	1	95
NEF 117	AcOH 25%	5	1	81
NEF 182	AcOH 25%	5	1	92
GAG 183	AcOH 80%	10	0.5	73
GAG 253	AcOH 25%	5	1	31
ENV	water	5	1	95



**TABLE 10**

Peptide	Solvent	Concentration (mg/ml)	Volume removed (ml)	Filtration yield (%) after mixing
NEF 66	AcOH 80%	20	0.250	quantitative
NEF 117	AcOH 80%	20	0.250	quantitative
NEF 182	AcOH 80%	20	0.250	quantitative
GAG 183	AcOH 80%	20	0.250	quantitative
GAG 253	AcOH 80%	20	0.250	quantitative
ENV	AcOH 80%	20	0.250	quantitative

\* to within the precision of the determination

**TABLE 11**

Peptide	Exact weight*** (mg)	Peptide net	Quantity expected* (µg per dose)	Quantity obtained*** (µg per dose)	yield** (%)
NEF 66	764	641	550	505 ± 15	89.14 - 94.6
NEF 117	739	641	550	621 ± 21	109.08 - 116.72
NEF 182	742	641	550	545 ± 16	96.23 - 102.05
GAG 183	741	642	550	478 ± 13	84.50 - 89.23
GAG 253	780	641	550	571 ± 28	98.76 - 108.95
ENV	810	642	550	593 ± 17	104.71 - 110.89

\* the target dose was 500 µg per peptide: an overdose of 10% was deliberately included at the time of weighing, given the yields obtained during the preparation of batch CK6

\*\* the yield ranges reflect the precision of the determination, and not a significant variation from one flask to another

\*\*\* the values in excess are due to imprecisions in the weighings of electrostatic powders by an operator wearing a standard pressure suit

**TABLE 12 - Test of uniformity of concentration**

	nef 66	nef 117	nef 182	gag 183	gag 253	env 303
	14.40	17.74	16.19	13.28	16.74	17.53
	14.38	17.75	16.21	13.27	17.27	17.63
	14.67	16.36	16.61	13.41	16.89	18.36
sample 1	14.49	17.28	16.34	13.32	16.97	17.84
	13.42	17.11	15.32	12.66	15.56	16.36
	13.81	17.04	15.32	12.67	15.89	16.51
	13.77	17.06	15.33	12.45	15.16	16.41
sample 2	13.67	17.07	15.32	12.59	15.54	16.43
	13.58	17.08	15.33	12.68	15.64	16.29
	13.70	17.08	15.31	12.62	15.82	16.28
	13.59	17.05	15.31	12.32	14.85	16.37
sample 3	13.62	17.07	15.32	12.54	15.44	16.31
	13.20	16.80	15.14	12.23	16.05	16.15
	14.53	17.34	15.74	13.06	15.93	17.17
	13.49	16.86	15.17	12.31	15.44	16.15
sample 4	13.74	17.00	15.35	12.53	15.80	16.49
	13.88	17.21	15.40	12.52	14.78	16.80
	13.94	17.17	15.39	12.59	15.20	16.72
	13.98	17.19	15.47	12.96	15.49	16.71
sample 5	13.94	17.19	15.42	12.69	15.16	16.74
	14.03	17.26	15.75	11.62	15.78	16.97
	13.99	17.20	15.73	11.39	15.77	17.02
	14.20	17.26	15.74	12.19	15.90	16.80

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**TABLE 12 - Test of uniformity of concentration (continued)**

sample 6	14.07	17.24	15.74	11.73	15.81	16.93
	13.78	17.29	15.67	12.69	16.13	18.04
	13.94	17.22	15.57	12.67	16.40	17.50
	13.95	17.23	15.55	12.28	16.19	17.37
sample 7	13.89	17.25	15.60	12.55	16.24	17.64
	13.84	17.06	15.38	12.50	15.62	17.90
	13.65	17.09	15.45	12.44	16.02	17.73
	13.73	16.94	15.37	nd	16.21	17.54
sample 8	13.74	17.03	15.40	12.47	15.95	17.73
	14.03	17.40	15.66	12.77	16.46	18.56
	14.07	17.33	15.72	11.92	16.61	18.41
	13.89	17.39	15.72	12.68	15.94	18.37
sample 9	14.00	17.37	15.70	12.46	16.34	18.45
	13.34	16.88	15.33	12.07	14.70	17.92
	13.71	17.24	15.66	12.36	15.12	18.50
	13.53	16.93	15.44	12.28	14.44	18.01
sample 10	13.53	17.02	15.48	12.24	14.76	18.14
	13.72	17.22	15.64	12.41	14.89	18.32
	13.75	17.33	15.72	12.36	14.82	18.31
	13.67	17.21	15.72	11.86	14.61	18.69
sample 11	13.71	17.25	15.69	12.21	14.77	18.44
	13.62	17.11	15.60	12.28	14.75	18.42
	13.74	17.13	15.70	12.44	14.98	18.31
	13.75	17.16	15.63	12.51	15.37	18.32
sample 12	13.70	17.13	15.65	12.41	15.03	18.35
	13.32	16.36	14.74	12.47	16.17	15.44
	13.31	16.41	14.68	12.51	16.26	15.56
	13.34	16.38	14.67	12.53	16.17	15.43

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**TABLE 12 - Test of uniformity of concentration (continued)**

sample 13	13.32	16.38	14.70	12.50	16.20	15.48
	13.76	16.72	14.75	12.67	16.10	15.59
	13.56	16.35	14.75	12.64	16.16	15.64
	13.60	16.67	14.76	12.64	16.06	15.64
sample 14	13.64	16.58	14.76	12.65	16.10	15.62
	13.36	16.40	14.53	12.48	15.90	15.64
	13.41	16.35	14.57	12.52	15.84	15.80
	13.44	16.43	14.50	12.46	15.77	15.60
sample 15	13.40	16.39	14.53	12.49	15.84	15.68
m	13.69	16.91	15.28	12.49	15.81	16.82
standard deviation	0.27	0.37	0.42	0.28	0.59	1.19
t's	0.58	0.79	0.89	0.59	1.24	2.51
min	13.11	16.12	14.39	11.91	14.57	14.30
max	14.27	17.70	16.18	13.08	17.05	19.33

5  $t = 2.110$  3 deviant values on sample 1 (probably dilution error)

Test of uniformity of concentration

Min accepted (-15%)	11.64	14.37	12.99	10.62	13.44	14.29
Max accepted (+15%)	15.74	19.45	17.58	14.37	18.18	19.34
Observed deviation	4.23%	4.67%	5.84%	4.70%	7.84%	14.95%

TABLE 13

anti-7 peptide CTL lines	Peptides recognized	Short peptides recognized
92102	GAG 246-281	
92105	NEF 125-147	
92109	NEF 101-126	NEF 101-110 NEF 116-126
	NEF 125-147	NEF 128-136
	NEF 155-178	NEF 169-178
	NEF 201-225	NEF 215-225
	GAG 246-281	
92120	GAG 246-281	
92125	NEF 155-178	NEF 169-178
92129	NEF 125-147 NEF 155-178 NEF 201-225	NEF 128-136 NEF 169-178 NEF 201-211 NEF 211-219
92117	negative	
92127	NEF 101-126 NEF 125-147 NEF 155-178	

**TABLE 14**  
Detection of specific antibodies of peptides of proteins NEF, GAG and ENV of the HIV virus, in the serum of volunteers immunized with a mixture of six lipopeptides

Volunteer <sup>a</sup>	Recovery period	Peptide recognized					
		N1	N2	N3	G1	G2	E
V4.6	W20	2.1	7.2	1.0	1.0	10.2	1.2
V4.15	W20	1.3	4.8	1.2	1.3	11.2	1.5
V4.16	W20	1.2	4.7	1.2	1.1	9.7	1.3
V4.17	W20	1.7	1.8	1.0	1.1	8.0	1.2
V4.18	W20	1.1	1.2	1.0	1.3	2.2	1.1
V4.28	W20	7.8	8.8	1.5	1	15	5.7
V4.1 (QS21)	W20	3.1	3.2	1.1	1.2	11.5	4.7
V4.5(QS21)	W20	1.2	4.2	1.3	1.1	8.1	2.1
V4.19 (QS21)	W20	1.2	5.3	1.2	1.3	5.1	3.8
V4.21 (QS21)	W20	1.2	4.7	1.0	1.2	9.3	1.9
V4.32 (QS21)	W20	6.6	14	1.8	1.9	21	4
V4.34 (QS21)	W20	7.1	21.2	1.2	1.8	36	8

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21)

<sup>b</sup> The serums of the volunteers were recovered before injection of the lipopeptides, and twenty weeks after. The three injections of the six lipopeptides were administered at 0.4 and 16 weeks.

<sup>c</sup> The detection of the specific antibodies of the peptides of the HIV virus was performed using an ELISA assay with serum dilution to 1/100. The ELISA assay plates were covered with NEF 66-97 (N1), NEF 117-147 (N2), NEF 182-205 (N3), GAG 183-214 (G1), GAG 253-284 (G2) or V3 ENV 303-335 (E).

TABLE 15  
Proliferative responses of the PBMC of the volunteers  
with lipopeptides NEF, GAG and ENV

Volunteer <sup>a</sup>	Recovery period	Proliferation index <sup>c</sup>			
		N1	N2	N3	G1
V4.6	W0	1.3	1.0	1.0	1.3
	W20	2.4	3.1(±1)	10(±7)	4.5(±1.1)
V4.15	W0	1.0	1.0	1.3	1.0
	W20	1.9	2.2	1.6	1.2
V4.16	W0	1.0	1.2	1.3	1.3
	W20	1.1	1.1	2.2	4.6(±0.6)
V4.17	W0	2.5	1.3	1.4	1.9
	W20	1.5	1.1	1.6	2.0
V4.18	W0	1.0	1.3	1.5	1.8
	W20	nd	nd	nd	nd
V4.28	W0	1.3	2.2	1.7	1.2
	W20	3.8(±0.6)	1.2	21(±2)	1.2

5

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

<sup>b</sup> The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

10 <sup>c</sup> 2 × 10<sup>5</sup> cells were cultivated with 1 µg/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

15 <sup>d</sup> The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1 µg/ml of PHA, PPD and SEB.



TABLE 15 continued (1)  
Proliferative responses of the PBMC of the volunteers  
with lipopeptides NEF, GAG and ENV

Volunteer <sup>a</sup>	Recovery period	Proliferation index <sup>c</sup>		
		G2	E	Proliferation induced by the culture medium <sup>d</sup>
V4.6	W0	1.0	1	871 ( $\pm 25$ )
	W20	7.2( $\pm 0.7$ )	3.6( $\pm 0.9$ )	280 ( $\pm 32$ )
V4.15	W0	1.4	1.5	1657 ( $\pm 182$ )
	W20	1.6	2.1	252 ( $\pm 30$ )
V4.16	W0	1.8	1.5	3830 ( $\pm 232$ )
	W20	3.6( $\pm 0.5$ )	3.9( $\pm 0.7$ )	1000 ( $\pm 168$ )
V4.17	W0	2.0	2.3	5708 ( $\pm 470$ )
	W20	2.0	2.8	1228 ( $\pm 54$ )
V4.18	W0	3.3( $\pm 0.7$ )	1.3	460 ( $\pm 49$ )
	W20	nd	nd	nd
V4.28	W0	1.2	1.2	869 ( $\pm 36$ )
	W20	8.2( $\pm 1.6$ )	7.6( $\pm 2.2$ )	2558 ( $\pm 186$ )

5

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

<sup>b</sup> The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

10 <sup>c</sup>  $2 \times 10^5$  cells were cultivated with 1  $\mu\text{g/ml}$  of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

15 <sup>d</sup> The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1  $\mu\text{g/ml}$  of PHA, PPD and SEB.

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TABLE 15 continued (2)  
Proliferative responses of the PBMC of the volunteers  
with lipopeptides NEF, GAG and ENV

Volunteer <sup>a</sup>	Recovery period	Proliferation index <sup>c</sup>			
		N1	N2	N3	G1
V4.1 (QS21)	W0	1.0	1.1	1.0	1.1
	W20	nd	nd	nd	nd
V4.5(QS21)	W0	1.5	1.6	1.5	1.6
	W20	4.6( $\pm$ 1.2)	3.1( $\pm$ 0.3)	1.5	2.0
V4.19 (QS21)	W0	1.0	1.1	1.2	1.1
	W20	24.3( $\pm$ 3.1)	8.5( $\pm$ 5)	4.4( $\pm$ 1.2)	3.1( $\pm$ 2.5)
V4.21 (QS21)	W0	1.3	1.3	1.2	3.9( $\pm$ 1)
	W20	6.5( $\pm$ 3)	1.4	2.3	2.8
V4.32 (QS21)	W0	1.0	1.0	1.1	1.3
	W20	1.0	1.0	1.7	1.2
V4.34 (QS21)	W0	1.0	1.1	1.1	1.2
	W20	3.4( $\pm$ 0.2)	1.1	3.3( $\pm$ 0.1)	2.2

5

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

<sup>b</sup> The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

10 <sup>c</sup>  $2 \times 10^5$  cells were cultivated with 1  $\mu$ g/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

15 <sup>d</sup> The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1  $\mu$ g/ml of PHA, PPD and SEB.

TABLE 15 continued (3)  
Proliferative responses of the PBMC of the volunteers  
with lipopeptides NEF, GAG and ENV

Volunteer <sup>a</sup>	Recovery period	Proliferation index <sup>c</sup>		
		G2	E	Proliferation induced by the culture medium <sup>d</sup>
V4.1 (QS21)	W0 W20	1.0 nd	1.0 nd	3107 ( $\pm$ 521) nd
V4.5(QS21)	W0 W20	1.5 3.9( $\pm$ 0.3)	1.3 5.0( $\pm$ 2.2)	341 ( $\pm$ 20) 776 ( $\pm$ 60)
V4.19 (QS21)	W0 W20	1.0 11.0( $\pm$ 2.7)	1.0 9.4( $\pm$ 2.8)	918 ( $\pm$ 102) 497 ( $\pm$ 168)
V4.21 (QS21)	W0 W20	1.3 11.3( $\pm$ 4)	1.4 3.3( $\pm$ 1.9)	322 ( $\pm$ 21) 1052 ( $\pm$ 82)
V4.32 (QS21)	W0 W20	1.2 10.1( $\pm$ 1.5)	1.2 0.9	4448 ( $\pm$ 75) 245 ( $\pm$ 30)
V4.34 (QS21)	W0 W20	1.2 4.4( $\pm$ 0.6)	1.2 3.1( $\pm$ 0.1)	5383 ( $\pm$ 309) 7381 ( $\pm$ 280)

5

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

<sup>b</sup> The PBMC of the volunteers were recovered before injection of the lipopeptides (W0), and during the twentieth week (W20).

10 <sup>c</sup>  $2 \times 10^5$  cells were cultivated with 1  $\mu$ g/ml of the HIV lipopeptides and the proliferation was measured by incorporation of tritiated thymidine at day 6. The lipopeptides were N1, N2, N3, G1, G2, and E. The proliferation index obtained with the culture medium only was equal to 1.

15 <sup>d</sup> The proliferative response (cpm) of the PBMC of the volunteers cultivated in the medium alone is given. All the PBMC samples proliferated in response to 1  $\mu$ g/ml of PHA, PPD and SEB.

**TABLE 16**  
Specificity of the CTL in the immunized volunteers

% of specific lysis of the cells

5

Lipopeptide incubated with target cells	V4.6 <sup>a</sup>		V4.16		V4.18		V4.28	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	70/1	70/1	50/1	50/1	80/1	80/1	10/1	10/1
None	5%	11%	2%	12%	8%	14%	5%	8%
NEF 66-97	9%	17%	8%	18%	11%	40%	12%	19%
NEF 117-147	9%	16%	2%	13%	6%	6%	19%	12%
NEF 182-205	4%	15%	2%	24%	6%	6%	2%	4%
E/T Ratio	70/1	70/1	30/1	30/1	55/1	55/1	10/1	10/1
None	4%	18%	5%	5%	8%	6%	2%	2%
GAG 183-214	7%	14%	9%	10%	nd	nd	2%	2%
GAG 253-284	9%	49%	11%	20%	6%	26%	2%	2%

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

10 <sup>b</sup> The target cells were autologous PBMC sensitized with 10 $\mu$ M of each of the lipopeptides, irradiated and marked with <sup>51</sup>Cr.

15 <sup>c</sup> The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).

<sup>d</sup> The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10<sup>3</sup> marked target cells, incubated with varying quantities of effector cells.

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**TABLE 16 (continued 1)**  
Specificity of the CTL in the immunized volunteers

% of specific lysis of the cells

5

Lipopeptide incubated with target cells	V4.5 (QS21)		V4.19 (QS21)		V4.21 (QS21)		V4.34 QS21)	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	100/1	100/1	60/1	60/1	40/1	40/1	35/1	35/1
None	16%	31%	2%	6%	2%	2%	10%	2%
NEF 66-97	10%	23%	0%	15%	2%	2%	2%	2%
NEF 117-147	18%	47%	2%	27%	2%	2%	2%	2%
NEF 182-205	10%	31%	0%	0%	2%	2%	2%	2%
E/T Ratio	140/1	140/1	60/1	60/1	46/1	46/1	35/1	35/1
None	23%	11%	0%	0%	2%	2%	2%	2%
GAG 183-214	21%	11%	0%	0%	2%	2%	2%	23%
GAG 253-284	20%	68%	0%	9%	2%	2%	5%	5%

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

10 <sup>b</sup> The target cells were autologous PBMC sensitized with 10 $\mu$ M of each of the lipopeptides, irradiated and marked with <sup>51</sup>Cr.

15 <sup>c</sup> The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).

20 <sup>d</sup> The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10<sup>3</sup> marked target cells, incubated with varying quantities of effector cells.

**TABLE 16 (continued 2)**  
**Specificity of the CTL in the immunized volunteers**

% of specific lysis of the cells

5

Lipopeptide incubated with target cells	V4.6 <sup>a</sup>		V4.16		V4.18		V4.28	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	70/1	70/1	25/1	25/1			10/1	10/1
None	3%	6%	32%	23%	nd	nd	2%	2%
V3 ENV 303-335	2%	36%	12%	49%	nd	nd	2%	17%
E/T Ratio			50/1	50/1			10/1	10/1
None			13%	23%			2%	2%
anti-A1			2%	48%			5%	34%
anti-A3			nd	nd			nd	nd

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

10 <sup>b</sup> The target cells were autologous PBMC sensitized with 10μM of each of the lipopeptides, irradiated and marked with <sup>51</sup>Cr.

15 <sup>c</sup> The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).

20 <sup>d</sup> The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10<sup>3</sup> marked target cells, incubated with varying quantities of effector cells.

**TABLE 16 (continued 3)**  
**Specificity of the CTL in the immunized volunteers**

% of specific lysis of the cells

5

Lipopeptide incubated with target cells	V4.5 (QS21)		V4.19 (QS21)		V4.21 (QS21)		V4.34 QS21)	
	W0	W20	W0	W20	W0	W20	W0	W20
E/T Ratio	60/1	60/1	60/1	60/1	86/1	86/1	35/1	35/1
None	22%	14%	0%	2%	7%	13%	5%	3%
V3 ENV 303-335	24%	16%	2%	6%	2%	23%	3%	3%
E/T Ratio							35/1	35/1
None							2%	2%
anti-A1							nd	nd
anti-A3							2%	2%

<sup>a</sup> The volunteers were immunized with six lipopeptides in the form of micelles, or with an adjuvant (QS21).

10 <sup>b</sup> The target cells were autologous PBMC sensitized with 10μM of each of the lipopeptides, irradiated and marked with <sup>51</sup>Cr.

15 <sup>c</sup> The chromium release assay was performed after three in vitro stimulations. The cytotoxic activity against autologous EBV cells incubated with the peptides or without peptides was measured in a release assay of 4 hours. The cytotoxic activity was considered as positive when the chromium release was 10% greater than that observed with the target cells alone. A1 and A3 correspond to EBV cells incubated with a group of peptides A1 (n 137-145, n 195-202, n 184-191, n 121-128 for V4.16 or n 183-191, n 121-128 for V4.28) and peptide A3 (n 73-82).

<sup>d</sup> The E/T ratio (ratio effector cells/target cells) corresponds to 5 x 10<sup>3</sup> marked target cells, incubated with varying quantities of effector cells.

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TABLE 17  
 CD8<sup>+</sup> T cells secreting  $\gamma$ -interferon/ex vivo assay  
 Number of cells secreting  $\gamma$ -interferon/ $1 \times 10^6$  cells

5

		V4.18 A2/11 B44/60		V4.5 (QS21) A2/11 B18/27		V4.21 (QS21) A1 B8	
		W0	W20	W0	W20	W0	W20
HLA-A1	NEF 121-128					1	41
	NEF 137-145					1	6
	NEF 184-191					6	26
	NEF 195-202					1	1
HLA-A2	NEF 136-145	0	0	0	4		
	NEF 190-198	nd	nd	4	16		
	GAG 183-191	nd	nd	2	16		
HLA-A11	NEF 73-82 <sup>c</sup>	0	15	0	0		
	NEF 84-92	0	55	nd	nd		
	EBN 416-424	500	500	66	63		
HLA-B8	NEF 90-97					1	51
	NEF 182-189					1	26
HLA-B27	NEF 134-141			2	14		
	GAG 263-272			0	9		
HLA-B18	NEF 135-143			0	9		

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CLAIMS

1. Mixed micelles or micro-aggregates for inducing an immune response containing at least :

- a first lipopeptide comprising at least one CTL antigenic determinant  
5 and at least one lipid unit, and
- a second lipopeptide comprising at least one helper T antigenic determinant and at least one lipid unit, which may be of a different type from the first lipopeptide unit.

2. Micelles or micro-aggregates according to claim 1, characterized in  
10 that the lipopeptides independently comprise one or more C<sub>4</sub>-C<sub>18</sub> lipid units.

3. Micelles or micro-aggregates according to one of claims 1 and 2, characterized in that the lipopeptides independently comprise one or two C<sub>4</sub>-C<sub>18</sub> lipid chains linked by a covalent bond to one or two amino acids of the peptide part.

4. Micelles or micro-aggregates according to one of claims 1 to 3,  
15 characterized in that the lipid units of the lipopeptides are composed of two palmitic acid chains linked to the NH<sub>2</sub> groups of a lysine.

5. Micelles or micro-aggregates according to one of claims 1 to 4,  
20 characterized in that the lipid units of the lipopeptides independently comprise a residue of palmitic acid, 2-aminohexadecanoic acid, oleic acid, linoleic acid, linolenic acid, pimelautide, trimexautide, or a derivative of cholesterol.

6. Micelles or micro-aggregates according to one of claims 1 to 5,  
characterized in that the non-lipid part of the lipopeptides, comprising the antigenic determinants, comprises between 10 and 100, and preferably  
25 between 10 and 50 amino acids.

7. Micelles or micro-aggregates according to one of claims 1 to 6, characterized in that the helper T antigenic determinant is a multivalent antigenic determinant.

8. Micelles or micro-aggregates according to one of claims 1 to 7,  
30 characterized in that the helper T antigenic determinant is the peptide 830-843 of the tetanus toxin with the following sequence:

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9. Micelles or micro-aggregates according to one of claims 1 to 7, characterized in that the helper T antigenic determinant is the antigenic determinant of hemagglutinin or the PADRE antigenic determinant.

10. Micelles or micro-aggregates according to one of claims 1 to 9, characterized in that the lipopeptides comprise at least one CTL antigenic determinant of a specific protein of melanoma, of a protein from HIV, from HBV, from papillomavirus, or protein p53, or a specific protein of *Plasmodium falciparum*.

11. Micelles or micro-aggregates according to one of claims 1 to 10, characterized in that they comprise the following lipopeptides:

GAG 17	E K I R L R P G G K K K Y K L K H I V K(Pam)-NH <sub>2</sub>
GAG 253	N P P I P V G E I Y K R W I I L G L N K I V R M Y S P T S I L D K(Pam)-NH <sub>2</sub>
POL 325	A I F Q S S M T K I L E P F R K Q N P D I V I Y Q Y M D D L Y K(Pam)-NH <sub>2</sub>
NEF 66	V G F P V T P Q V P L R P M T Y K A A V D L S H F L K E K G G L K(Pam)-NH <sub>2</sub>
NEF 116	H T Q G Y F P D W Q N Y T P G P G V R Y P L T F G W L Y K L K(Pam)-NH <sub>2</sub>
TT	Ac-Q Y I K A N S K F I G I T E L K K K(Pam)-NH <sub>2</sub>

12. Micelles or micro-aggregates according to one of claims 1 to 10, characterized in that they comprise the following lipopeptides:

LSA3 CT1	L L S N I E E P K E N I I D N L L N N I K(Pam)-NH <sub>2</sub>
LSA3 NRI	Ac-D E L F N E L L N S V D V N G E V K E N I L E E S Q K(Pam)-NH <sub>2</sub>
LSA3 NRII	Ac-L E E S Q V N D D I F N S L V K S V Q Q E Q Q H N V K(Pam)-NH <sub>2</sub>
LSA3 RE	K(Pam) V E S V A P S V E E S V A P S V E E S V A E N V E E S V A E N V-NH <sub>2</sub>

13. Use of micelles or micro-aggregates according to one of claims 1 to 12 for the production of a drug or a vaccine for inducing a specific immune response.

14. Use of micelles or micro-aggregates according to one of claims 1 to 12 for the production of a drug or a vaccine for inducing a specific immune response against HIV, HBV, papillomavirus, p53, melanoma or malaria induced by *Plasmodium falciparum*.

15. Pharmaceutical composition characterized in that it comprises a pharmacologically effective dose of micelles or micro-aggregates according to one of claims 1 to 12 and pharmaceutically compatible vehicles.

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17. Method for producing micelles or micro-aggregates according to one of claims 1 to 12, comprising the following steps:

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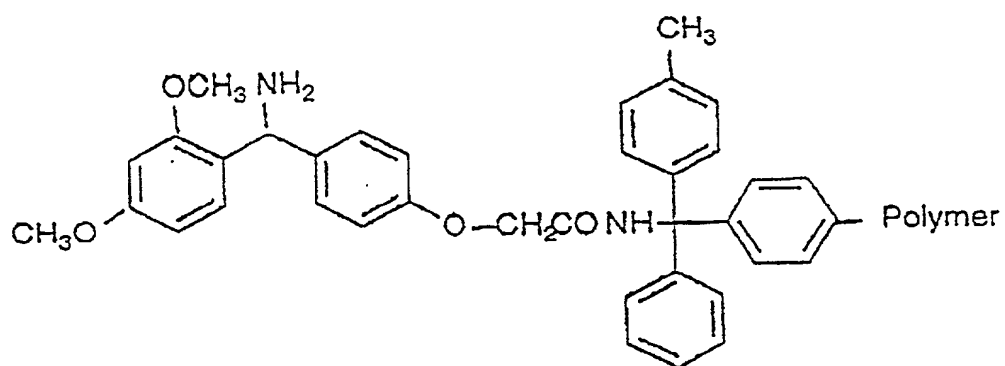
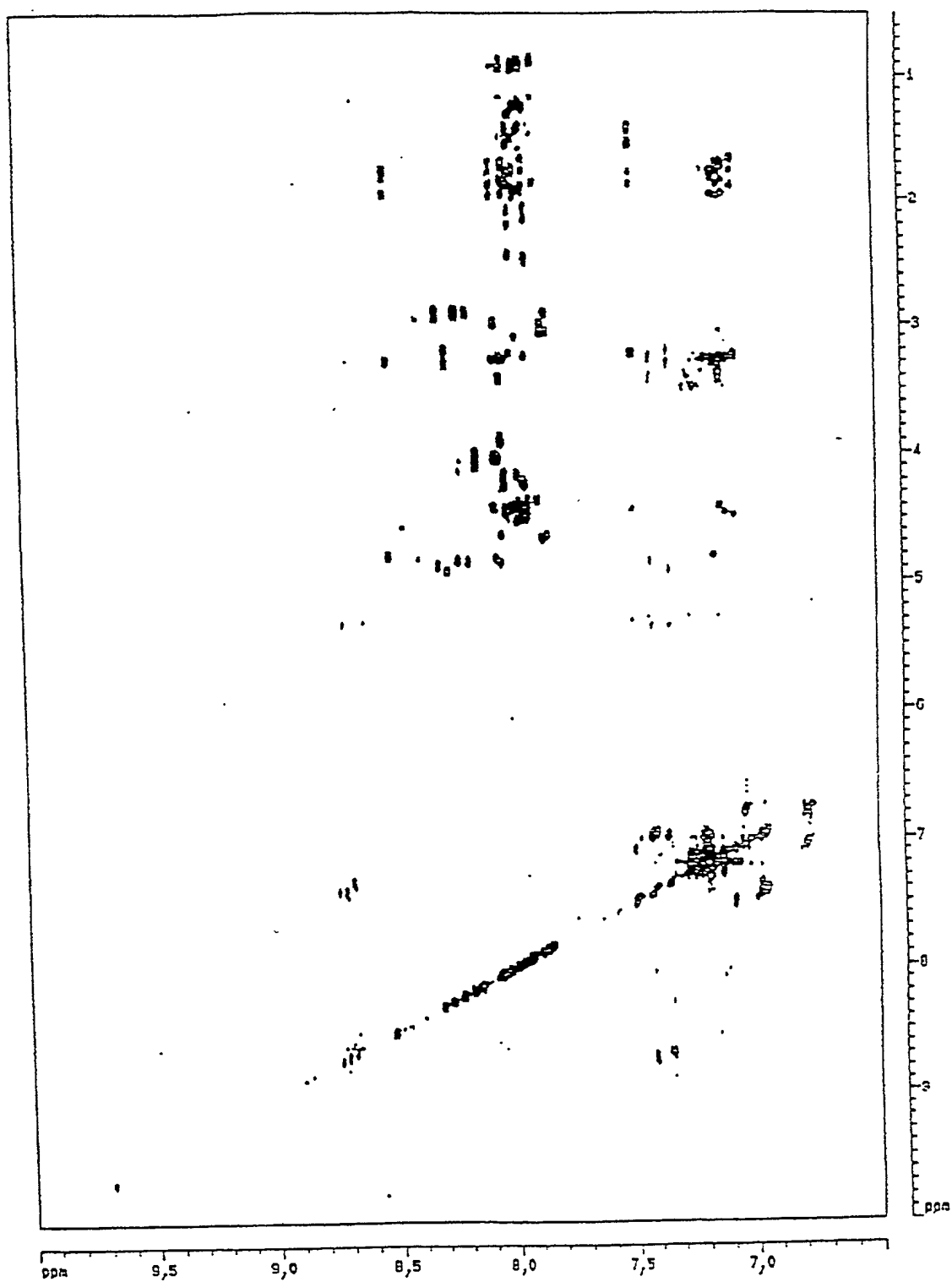


FIGURE 1

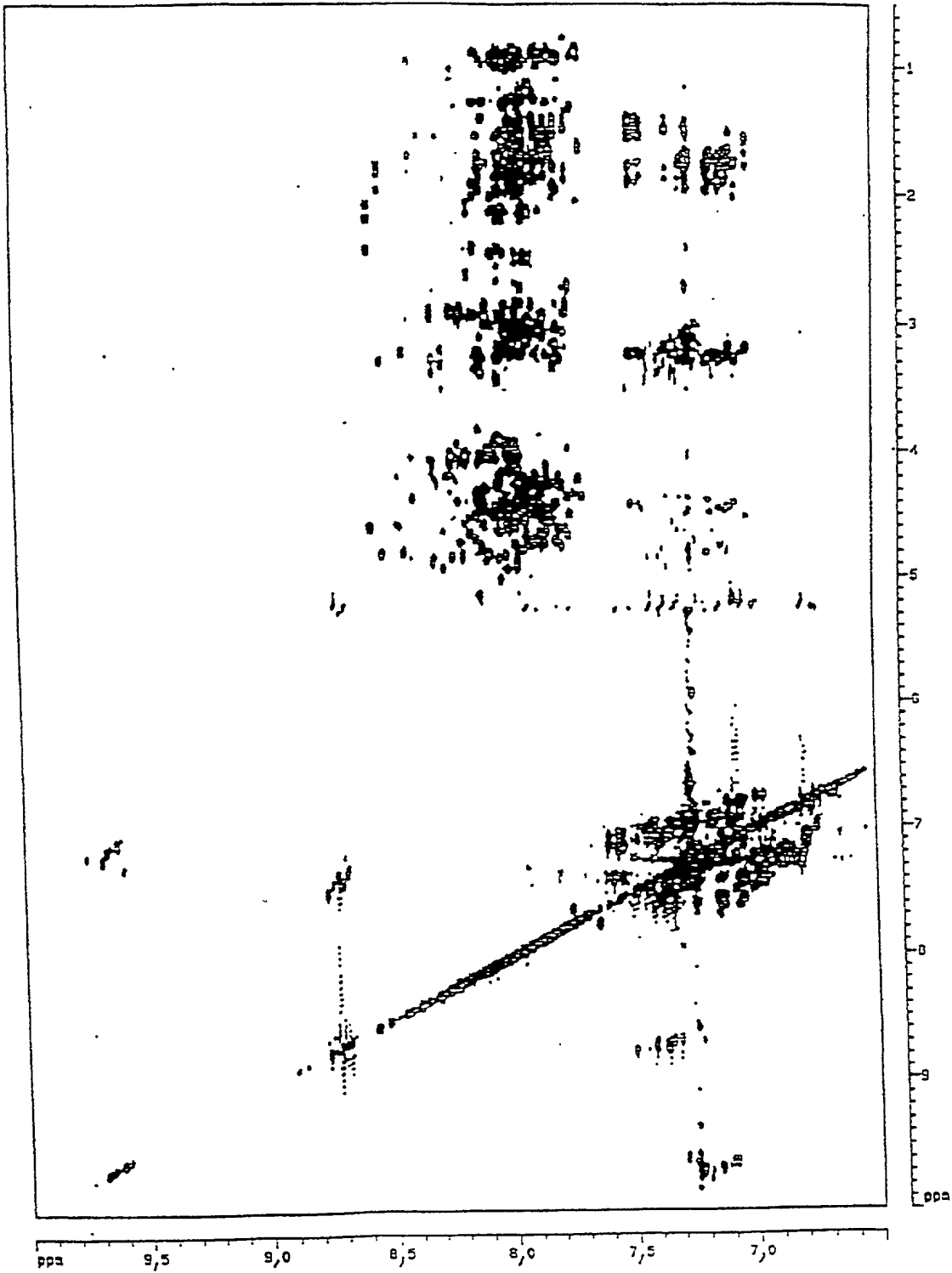
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0955780.11700

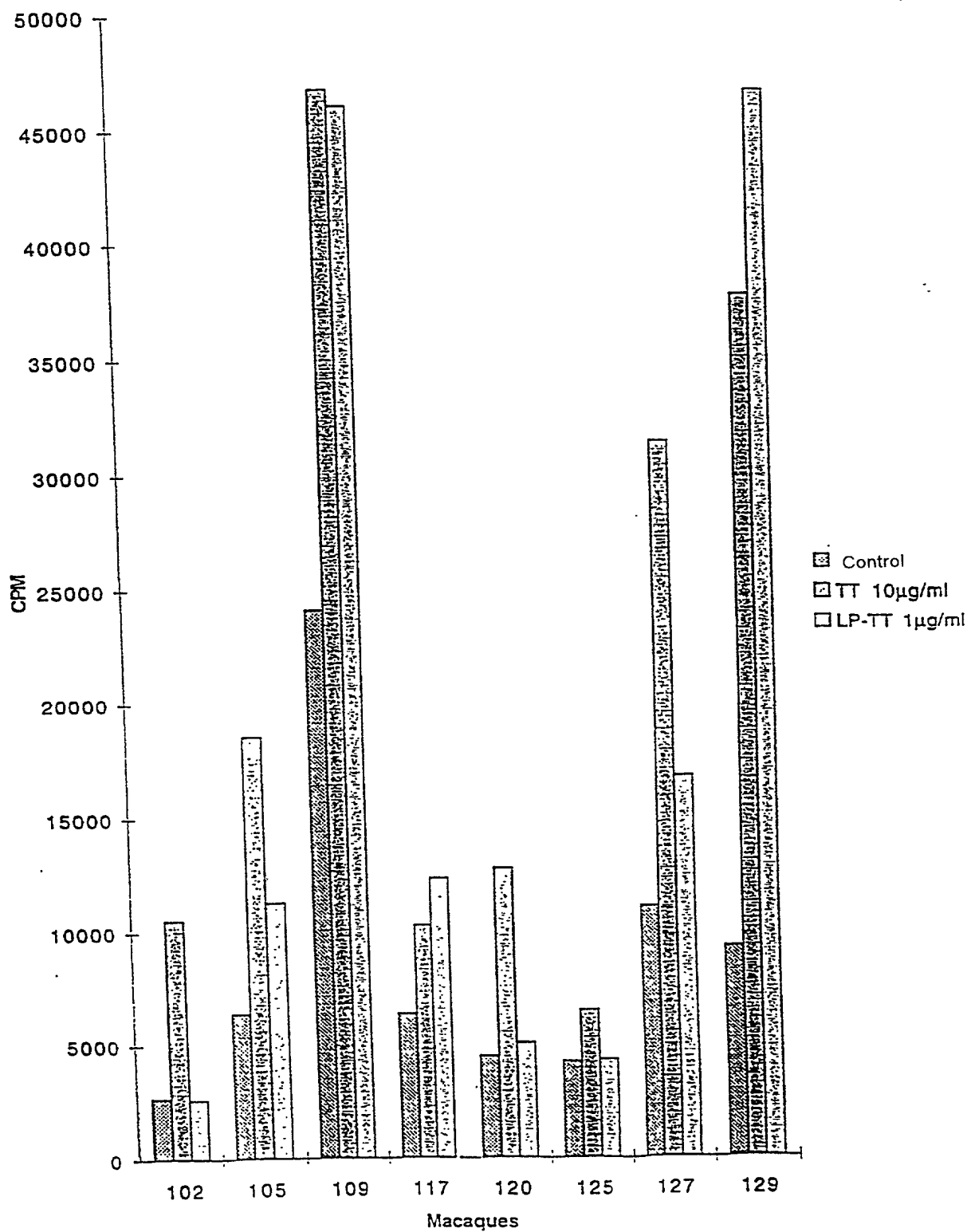


3/11

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4/11



5/11

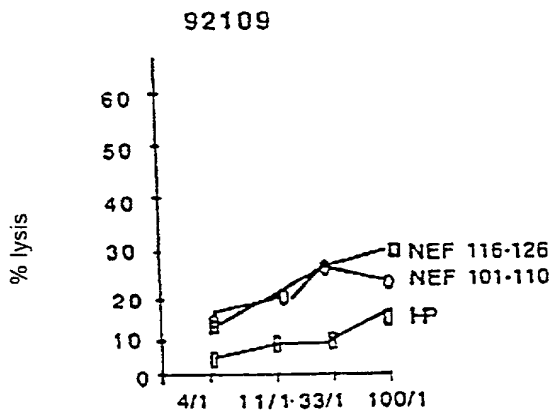


FIGURE 5A

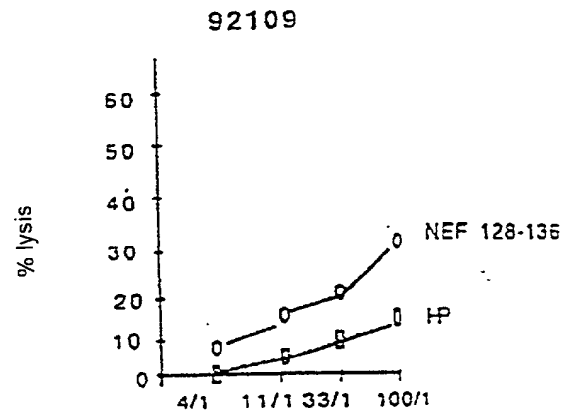


FIGURE 5B

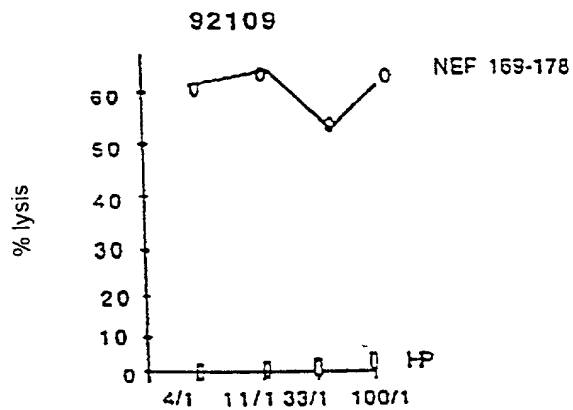


FIGURE 5C

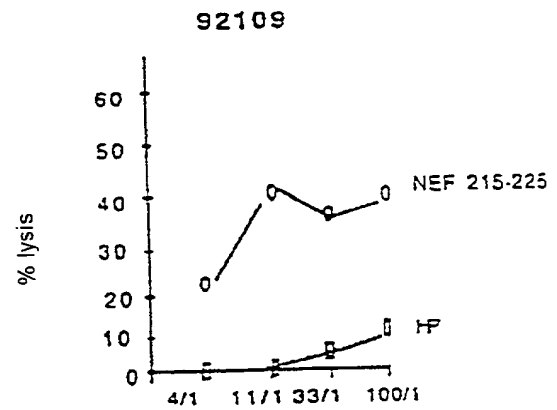


FIGURE 5D



6/11

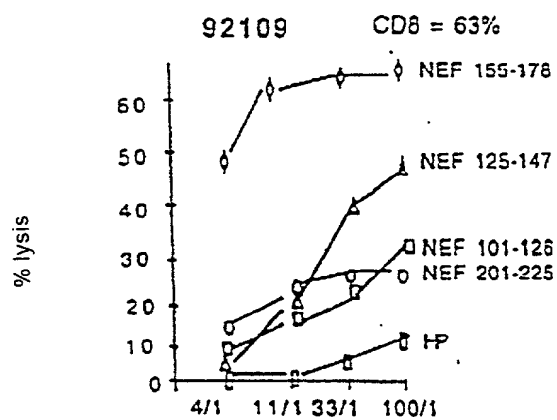


FIGURE 5E

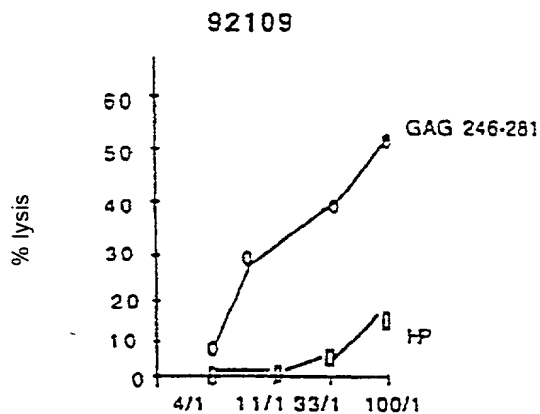


FIGURE 5F

7/11

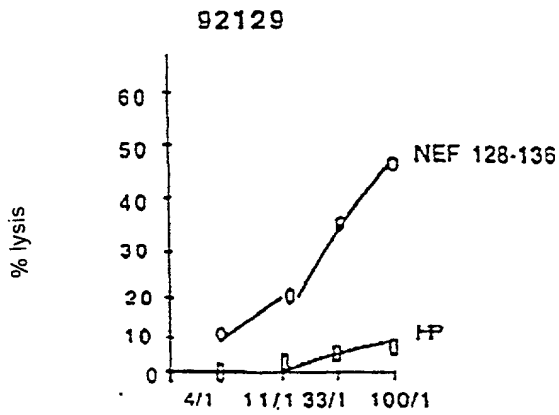


FIGURE 6A

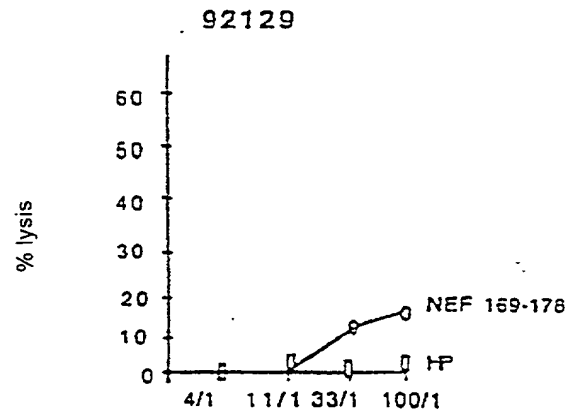


FIGURE 6B

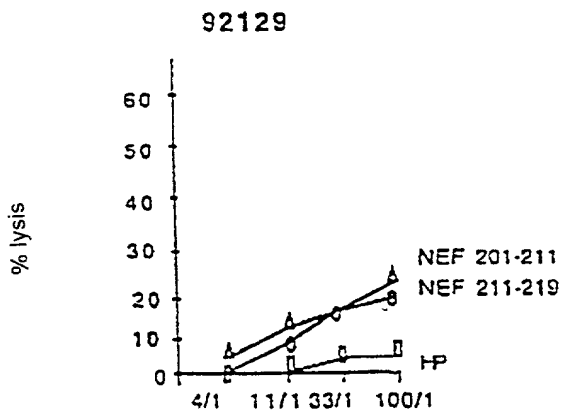


FIGURE 6C

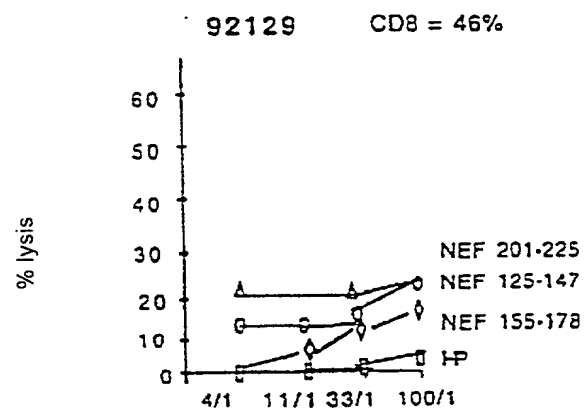


FIGURE 6D

8/11

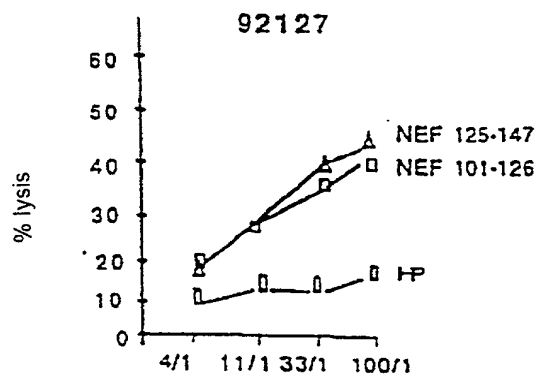


FIGURE 7A

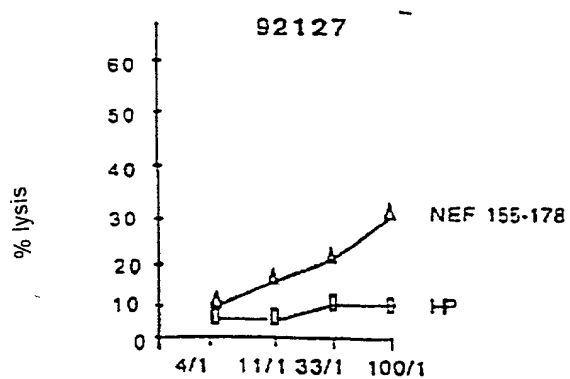


FIGURE 7B

9/11

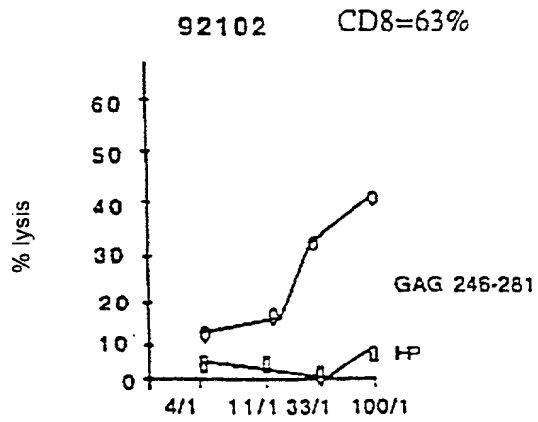


FIGURE 8

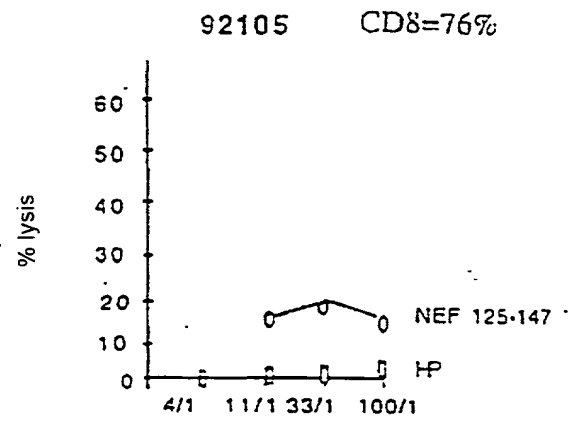


FIGURE 9

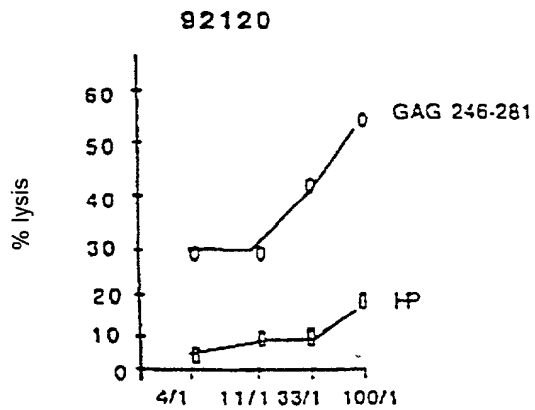


FIGURE 10

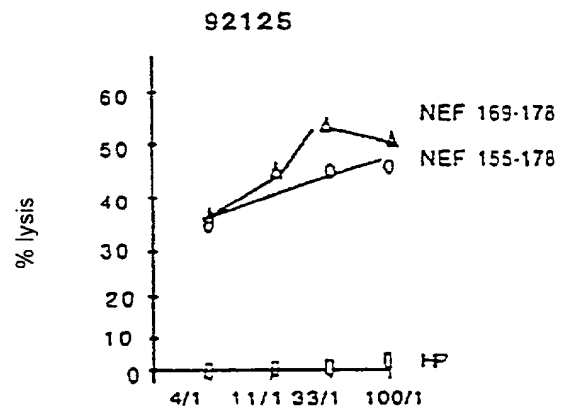


FIGURE 11

10/11

Fig. 12C

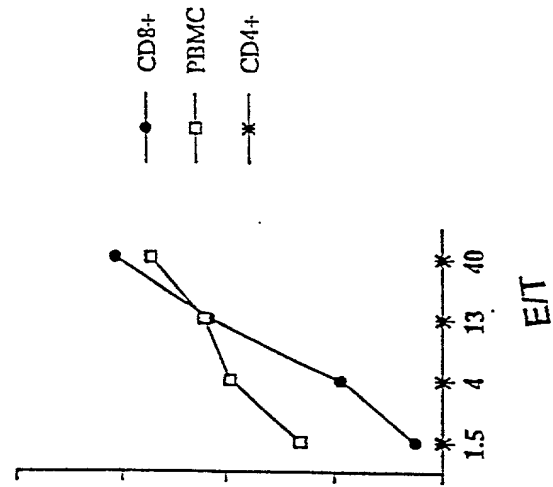


Fig. 12B

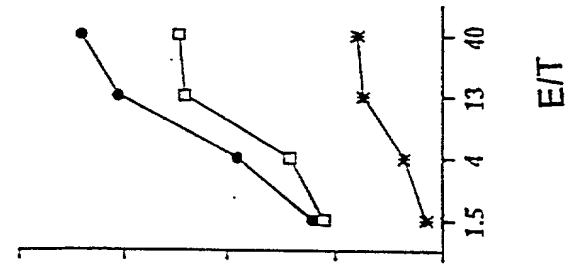
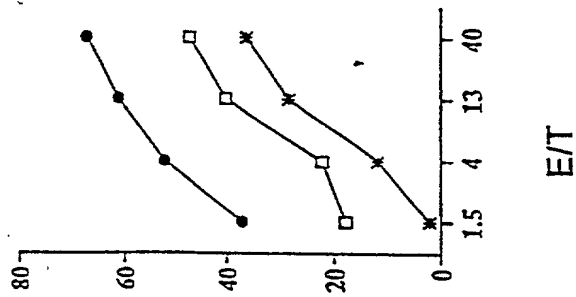


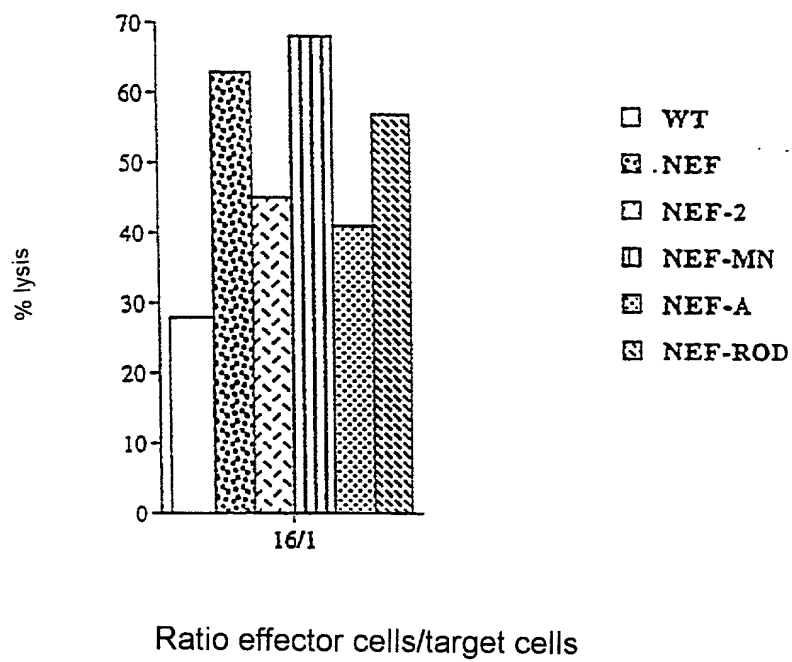
Fig. 12A



% specific lysis

11/11

FIG. 13



Docket No.  
1091-~~8~~ PCT/US

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Mixed lipopeptide micelles for inducing an immune response and their therapeutic uses.

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 2nd December 1998 as United States Application No. or PCT International

Application Number PCT/FR98/02605

and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

<u>97 15246</u>	<u>FRANCE</u>	<u>3rd December 1997</u>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	
<u>                    </u>	<u>                    </u>	<u>                    </u>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	
<u>                    </u>	<u>                    </u>	<u>                    </u>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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Citizenship French	
Post Office Address see above	

Full name of eighth inventor, if	
Eighth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of ninth inventor, if any	
Ninth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	

Full name of tenth inventor, if any	
Tenth inventor's signature	Date
Residence	
Citizenship	
Post Office Address	